Using genetic analysis to determine the effects of disease on the spatial dynamics of the invasive fall armyworm, *Spodoptera frugiperda*, in Africa.

Amy Jane Withers BSc MRes

PhD Thesis

December 2021

Using genetic analysis to determine the effects of disease on the spatial dynamics of the invasive fall armyworm, *Spodoptera frugiperda*, in Africa.

Amy Jane Withers BSc MRes

2021

A thesis is submitted to Lancaster University in fulfilment of the requirements for the degree of Doctor of Philosophy.

Project Supervisors

Professor Kenneth Wilson
Lancaster Environment Centre, Lancaster University
Dr Christopher M. Jones
Liverpool School of Tropical Medicine
Dr Judith A. Smith
University of Central Lancashire
Dr Aislinn J. Pearson
Rothamsted Research

Thesis Abstract

Using genetic analysis to determine the effects of disease on the spatial dynamics of the invasive fall armyworm, *Spodoptera frugiperda*, in Africa.

Amy J. Withers

Invasive crop pests are a global problem, resulting in food shortages, severe economic burdens, and devastating environmental losses. Many invasive insects are migratory with strong flight ability which further enables their rapid spread. Disease can control pests through occurring naturally and as biocontrol agents. However, the presence of naturally occurring diseases and potential flight routes of invasive pests are unknown when an invasive species first reaches an area. This thesis uses a combination of field collections, molecular biology, and behavioural experiments to fill some key knowledge gaps in our understanding of the invasive fall armyworm (*Spodoptera frugiperda*) in Africa. The fall armyworm is a migratory crop pest that first arrived in Africa in 2016 and rapidly spread across the continent. Chapter One provides a general introduction to the topics covered in this thesis, focusing on migratory insects to explore what is currently known about pest control, how disease interacts with migration and what genetic analysis can tell us about these topics. Chapter Two, uses fall armyworm larvae collected in six African countries to identify the presence of microbial natural enemies. It then models how environmental factors may influence the distribution and prevalence of the viral natural enemy, *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV). To improve current understanding of population structure of the fall armyworm in Africa, Chapter Three uses a combination of molecular approaches, and investigates whether this can explain variation in the distribution of SfMNPV. Chapter Four takes an in-depth look at the effects SfMNPV has on the fall armyworm during flight, using RNA sequencing to reveal what occurs at the molecular level that could lead to changes in migratory capacity. It compares the molecular responses of males and females, highlighting similarities and differences across many key areas including metabolism, immunity, and reproduction. Using four genes identified by RNA sequencing, Chapter Five focuses on gene expression of the immunity related Toll-pathway. This furthers our knowledge of how the fall armyworm responds to SfMNPV and flight and helps to unravel why males and females respond differently. Finally, Chapter Six is a general discussion chapter that brings these findings together to discuss and consolidate the knowledge that this research has contributed to the field. Overall, this thesis greatly contributes to current understanding of fall armyworm in Africa, and what molecular changes are triggered by flight and disease in a migratory insect pest.

Contents

Tables

Figures

Acknowledgements

Without my brilliant supervisors this PhD could not have been completed, so firstly, Ken, Chris, Aislinn and Judith, thank you to for always pushing me to do better, guiding me to find alternatives when experiments weren't working and for helping me find a way through the maze that is a PhD. Ken especially, for patiently correcting my pronunciations and inconsistencies, and for the amazing opportunities you have given me, including the first, and probably the only time, I'll ever do karaoke. Chris for enthusiastically encouraging all my experiment ideas, *for example*, searching shops in Malawi to find a way to collect rain. Aislinn, for being an excellent mentor and for teaching me many lab skills. Judith, for helping me to unravel the mysteries of molecular biology. I am so grateful for the support and advice I received from you all.

To everyone, past and present, in the Insect and Pathogen Ecology Group, especially Annabel, Phill and Philip, for helping raise thousands of moths and answering my many questions. Annabel, without your helpfulness then this work would not have been possible, thank you.

To Donald, Gilson, Patrick, Bellancile, Sevgan, Kentosse, Miyanda, Guillaume and many others who helped make this possible by collecting and sending fall armyworm, thank you.

Siân, for being my best friend for over 10 years and always being there for me.

And, finally, without the endless support of my amazing family I wouldn't have been able to do a PhD.

Mom and Dad, for all you have done for me, but a special thank you for always encouraging me to do what I wanted, from letting me be that weird kid that spent hours catching insects to try to keep as pets, to changing my mind frequently about what I wanted to do.

Becky, for simply being the best big sister in every possible way.

Nan and Grandad, for the constant love and support.

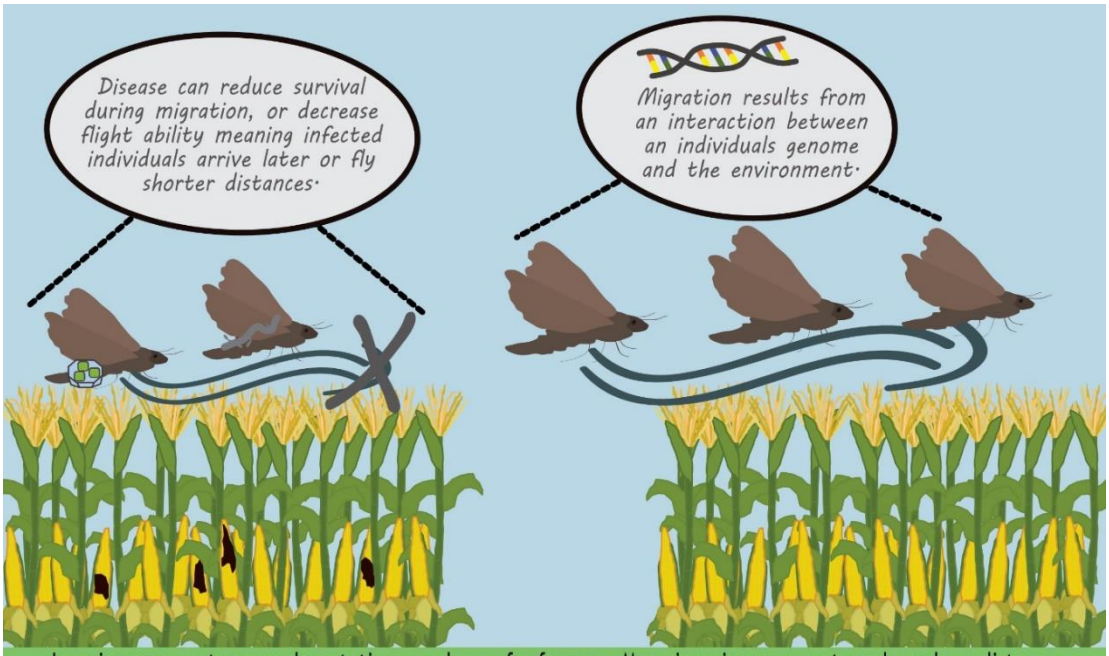
Jon, for everything. For understanding when plans were rearranged by moths, the long walks (especially for agreeing that walking 186 miles is an excellent way to spend a holiday!), the many one-way stats conversations, the pineapple plant, listening repeatedly to every science presentation I have done, and so much more. Thank you for being right by my side at every step of the way.

Declaration and Funding Statement

I declare that the work presented in this thesis is my own, except where acknowledged, and has not been submitted for the award of Doctor of Philosophy elsewhere.

This work is funded by Natural Environment Research Council through the Envision Doctoral Training Programme (NE/L002604/1), and was awarded to Professor Kenneth Wilson, Dr Christopher M. Jones, and Amy J. Withers to be carried out as a partnership between Lancaster University and Rothamsted Research.

Chapter 1 - Invasive species, migration, disease, and genetic analysis; how are they linked and why are they important for the fall armyworm?



Invasive crop pests cause devastating crop losses for farmers. Many invasive crop pests undergo long-distance migratory flights. This enables the pest to survive year-round, and reach favourable habitats for reproduction. Migration occurs as an interaction between the environment, genome and individual. Pathogens have an important role in these interactions and are a key driver behind the evolution of migration

1.1 Migratory insects as invasive crop pests

Invasive species are those that have been introduced into a new, non-native range that have then established as a species, able to survive and reproduce. There are many problems associated with invasive species. They can have devastating effects on ecosystems, such as through altering food webs, changing nutrient availability and reducing biodiversity (Vitousek et al., 1997; Ehrenfeld, 2010; Bellard et al., 2016). This can result in the extinction of native species and change the behaviour of keystone species (Vitousek et al., 1997; Ehrenfeld, 2010; Bellard et al., 2016). Furthermore, throughout the world invasive species have huge economic burdens for the countries they have invaded through their impact on agriculture, human health, and infrastructure (Pimentel et al., 2001; Peh, 2010; European Environment Agency, 2012; Bradshaw et al., 2016; Pratt et al., 2017).

The introduction of invasive species can occur in three ways; intentional, accidental, or natural spread from a previous introduction (Kimberling, 2004; Snyder and Evans, 2006). Some species are intentionally introduced and then become invasive, this has been widely observed following the introduction of the ladybird (*Harmonia axyridis*) for pest control (Roy et al., 2016). Accidental introductions are those that occur through human trade or travel, for instance, the introduction of the brown marmorated stink bug (*Halyomorpha halys*) into Italy from North America or Asia through trade (Cesari et al., 2015) and of mosquitos (*Aedes aeqypti* and *Aedes albopictus*) worldwide through tyre trade including the USA, Panama and South Africa (Reiter and Sprenger, 1987; Jupp and Kemp, 1992; Bennett et al., 2019). Trade of products, particularly crops, is considered to be the leading pathway for the introduction of invasive species (Brenton-Rule et al., 2016). The chances of an invasive species being introduced varies between countries due to many factors including the type of produce, amount of forest cover and within country regulations (Brenton-Rule et al., 2016). However, the overall risk of invasive pests being introduced to a new country via fruit and vegetables imports is relatively low (Lichtenberg and Olson, 2018). Finally, once an invasive species has been introduced and established, it can spread naturally into new areas. This occurred in the Americas following introduction of the cotton bollworm (*Helicoverpa armigera*) into South America in 2008, it rapidly spread through Central America using long-distance flight (Kriticos et al., 2015; Jones et al., 2019).

When invasive species are first introduced to a new region they often undergo a rapid increase in abundance and range. One of the possible reasons for this is described by the *enemy release hypothesis*, which assumes that the newly introduced species has escaped

its natural predators, competitors, and diseases, thus enabling it to rapidly increase and spread (Cornell and Hawkins, 1993; Keane and Crawley, 2002; Roy et al., 2011). However, the real-world applications of this theory are hard to test, particularly in insects, and many studies assume the proliferation is due to an escape from natural enemies but cannot establish a direct cause and effect relationship (Roy et al., 2011).

The largest group of invasive animals are insects, and many have the key traits associated with rapid spread and quick establishment, leading to an estimated 7500 invasive arthropod species throughout the world (Pimentel et al., 2001). These traits include high phenotypic plasticity so they can survive in a wider range of environments and r-selected life histories (i.e., high fecundity and fast growth rates) (Sakai et al., 2001; Davidson et al., 2011). Another key factor in the success of many invasive insect species is that they are usually generalist predators or herbivores that can survive on a wide variety of food sources. This enables them to exploit more habitats, in which they are can out-compete native species (Snyder and Evans, 2006; Crowder and Snyder, 2010).

Many highly invasive insects are migratory, this facilitates their rapid spread through new regions. This migratory behaviour can lead to invasive crop pests becoming very widespread in their new regions, as observed in the cotton bollworm which rapidly colonised most of South and Central America following its introduction (Jones et al., 2019). However, migratory invasive pests may be able to expand their distribution in their invasive region but there is no guarantee that they will become established due to constraints including host plant availability, competition, or climate suitability (Leung et al., 2012; Keller et al., 2011). The invasive island sugarcane planthopper (*Eumentopina flavipes*) has wind-assisted migration that theoretically allows it to spread further than its known range, this discrepancy between theoretical and observed range is likely due to habitat restrictions (e.g., host plant availability, inter-species competition) preventing colonisation once it has arrived (Anderson et al., 2010).

Invasive insect species cause many problems, including agricultural losses that result in huge economic burdens and food shortages. The economic burden of invasive arthropods on crops throughout the world is estimated to be around $44.1 billion (Pimentel et al., 2001). Certain parts of the world are at greater risk from invasive species than others, with the most at risk areas in terms of food production located in sub-Saharan Africa where there is a high dependency on agriculture, both economically and for food security (Paini et al., 2016; Sileshi et al., 2019). The spotted stem borer (*Chilo partellus*) can result in maize yield losses

of up to 33% in countries such as Kenya, Uganda and Ethiopia (Paini et al., 2016), and invasive leaf miners (*Liriomyza huidobrensis, L. sativae, L. trifolii*) cause an average pea yield loss of 31 – 66% across Kenya (Gitonga et al., 2010). Consequently, one reason for this risk is that many invasive crop pests in Africa target staple foods grown by small holders.

Overall, there are many factors that increase the likelihood of a species becoming invasive if it is introduced to a new region, including habitat differences (e.g., fewer natural enemies) as well as behavioural traits (e.g., being generalists). Agriculture is seriously affected by invasive crop pests, hence it is important to understand invasive species in more detail as they reach new regions. Understanding an invasive species life-history, behaviour and natural enemies can help control the pest and mitigate against crop losses.

1.2 Control of invasive crop pests

Control of insect pests is vitally important to help reduce yield losses and grow more food on less land, as well as mitigate against food shortages and the economic impact caused. If done sustainably, pest control can have the potential to make a significant contribution to achieving the United Nations Sustainable Development Goals of reducing food shortages, improving food security and promoting sustainable agriculture (United Nations, 2020).

One of the primary problems with invasive species is that when first introduced they often go unnoticed, or are not officially reported, for a prolonged period. This allows sufficient time for populations to become established and spread, whereas early identification and control can be vital in preventing the invasive species from establishing. For instance, the brown marmorated stink bug (*Halyomorpha halys*) was first sighted in Pennsylvania in 1996, however, it was not officially recorded as being present until 2001, and it has since spread to 41 states (Hoebeke and Carter, 2003; CABI, 2021). Even once an invasive species has established, it may go unrecorded for a prolonged period because many farmers can struggle to identify the insect or the damage symptoms in the crop (Gitonga et al., 2010). To counter this, multiple methods of early detection are being used and developed throughout the world, including eDNA screens, citizen science programs, photo recognition applications and trap monitoring schemes (Crall et al., 2010; Maistrello et al., 2016; Valentin et al., 2018; FAO, 2021; GO-Micro, 2021).

The challenge of managing invasive insect pests fits in to the wider issue of sustainable land use. Integrated pest management (IPM) takes a more agro-ecological approach to controlling insect populations, using a combination of different cultural and

ecological control methods to successfully control pests with pesticides being used only as a last resort (Ehler, 2006). This can be effective, with the combination of multiple control methods leading to a reduction in crop losses as well as an increase in profit and food security (Rejesus and Jones, 2020). Many studies have shown that using IPM in Africa could lead to significant increases in crop yields, with the greatest benefit seen when intercropping and push-pull systems were used (Pretty and Bharucha, 2015; Himmelstein et al., 2017). The push-pull system is when alternative hosts for the pests are planted around the border of the main crop, and the main crop is intercropped with plants that repel the pest (Pyke et al., 1987). This pulls the pest towards the alternative host and pushes them away from the crop, so the crop is not targeted by the pest resulting in reduced yield losses. This method can be highly successful and it has effectively worked in East Africa to protect maize against the invasive fall armyworm, resulting on an average of 87% less plant damage compared to the control, monocrop plots (Midega et al., 2018).

Yet IPM uptake has been slow in much of Africa, particularly among smallholders, and possible reasons for this include limited training for farmers, insufficient policy support or incentives, and a lack of research (Himmelstein et al., 2017; Parsa et al., 2014; Muriithi et al., 2020). The reasons driving slow IPM uptake in Africa vary between countries, as is the case for IPM uptake for control of the invasive Tephritid fruit fly (*Bactrocera dorsalis*) on mango crops in Kenya and Ethiopia, with farmers in Kenya more likely to use IPM than in Ethiopia (Muriithi et al., 2020). In Kenya, the likelihood of using IPM increased with factors such as younger household heads and if livestock was owned, whereas in Ethiopia likelihood increased for larger farms and if government support offices were closer (Muriithi et al., 2020).

In Africa, the most widely used control method are insecticides, which can be used against a wide variety of pests (Badenes-Perez and Shelton, 2006; Kalule et al., 2006; Gitonga et al., 2010). However, many invasive species are resistant to multiple insecticides which reduces or even eliminates their effectiveness, as has been observed in the fall armyworm (*Spodoptera frugiperda*), the cotton bollworm (*Helicoverpa armigera*), and the tomato leaf miner (*Tuta absoluta*) (Buès et al., 2005; Guedes et al., 2019; Zhang et al., 2020). Insecticides pose a serious health risk to many small holders in Africa due to high levels of insecticide misuse, both in how they are stored and by wearing insufficient personal protective equipment during spraying (Jepson et al., 2014; Lekei et al., 2014; Isgren and Andersson, 2020; Andersson and Isgren, 2021). Though pesticides are widely used due to their ease of use and low cost in relation to alternative methods, they are often prohibitively expensive

for many small holders. Consequently, they are not always an option for control, or doses below the recommended are used reducing their efficiency (Gitonga et al., 2010; Midega et al., 2012; Jepson et al., 2014).

Considering the issues associated with over-reliance on insecticides, it is becoming increasingly common to encourage farmers to move away from insecticide use and rely on safer, and often cheaper, methods of pest control.

1.2.1 Biocontrol of invasive crop pests

Another alternative to insecticides is biocontrol, which is when live insects or microorganisms are used to protect against crop pests. Biocontrol has advantages over other methods of control, especially when used in combination with other sustainable methods as part of IPM. These advantages include safer storage and application for farmers, target specificity so limited effects on non-target species, long-term sustainability due to no development of resistances and reduced pesticide use (van Lenteren et al., 2018; Lacey et al., 2015b).

Host-specific parasitoids can be highly beneficial for controlling insect pests, and the mass rearing and release of these parasitoids can lead to significant reductions in invasive crop pests. The parasitoid *Comperiella calauanica* reduces levels of the invasive coconut scale insect (*Aspidiotus rigidus*) in the Philippines, and the parasitoid *Apoanagyrus lopezi* helps protect cassava from the invasive cassava mealybug (*Phenacoccus manihoti*) in Africa (Neuenschwander, 2001; Almarinez et al., 2020). As well as parasitoids, predatory insects are commonly used as biological control agents, such as the use of the predatory mite, phytoseiid (*Typhlodromalus aripo*) to reduce the invasive cassava green mite (*Mononychellus tanajoa*) in Africa (Zannou et al., 2007). Reduced insecticide use and limited negative effects on non-target species are just a few of the many advantages of using parasitoids and predators as biological control mechanisms (Neuenschwander, 2001; Zannou et al., 2007). However, there is the risk that the introduced biocontrol agent could become an environmental problem itself. This happened following the introduction of the ladybird (*Harmonia axyridis*) for pest control in many countries, including the USA and UK, where it is now outcompeting native ladybird species (Roy et al., 2016).

Microorganisms as biocontrol agents can be much more effective than insecticides, and are utilised in classical, conservation and augmentative biocontrol (Lacey et al., 2015b). There are many species options for biocontrol, including nematodes, bacteria, nucleopolyhedroviruses, granuloviruses and *Metarhizium* and *Beauveria* fungal species

(Srinivasan et al., 2019; Lacey et al., 2015b). The effects of biological control agents can be highly beneficial in controlling invasive crop pests; in Senegal, insecticides did not reduce damage against the tomato pinworm, whereas the bacterial biopesticide *Bacillus thuringiensis* (*Bt*) significantly reduced crop damage (Sarr et al., 2021).

Nucleopolyhedroviruses (NPV) are highly species-specific viruses that target a wide range of Lepidopteran insect pests. NPV are popular as biocontrol agents due to a number of traits; they are capable of causing virulent disease in the target host, spreading vertically and horizontally, and form occlusion bodies that can help protect the virus (Fuxa, 2004). NPV occur naturally in wild populations of many species, including economically damaging pest species like the fall armyworm (*Spodoptera frugiperda*), African armyworm (*Spodoptera exempta*) and the Indian gypsy moth (*Lymantria obfuscata*) (Brown and Swaine, 1965; Virgen et al., 2013; Gupta et al., 2016). Furthermore, NPV have been developed commercially and products are available for the control of key Lepidopteran crop pests including the cotton bollworm (*Helicoverpa armigera*), beet armyworm (*Spodoptera exigua*) and velvetbean caterpillar (*Anticarsia gemmatalis*) (Lacey et al., 2015b). NPV can be highly effective biological control agents. In field trials using *Spodoptera exempta nucleopolyhedrovirus* (SpexNPV) led to reductions in African armyworm larvae of up to 77%, and *Lymantria obfuscata nucleopolyhedrovirus* (LyobMNPV) reduced the Indian gypsy moth by up to 63% (Grzywacz et al., 2008; Gupta et al., 2016).

Despite the numerous benefits of biocontrol, many farmers in Africa do not use them, with as few as 10% of farmers in Kenya reporting that they use biocontrol methods (Constantine et al., 2020). This low use of biopesticides is due to a number of factors including lack of awareness, prohibitive costs, accessibility, or perceiving them as less effective than other control methods (Constantine et al., 2020).

1.2.2 Natural enemies in the control of invasive crop pests

Biological control agents do not always have to be introduced from the native host range as natural enemies can act as a source of biological control. Natural enemies are predators or pathogens that occur naturally within a region that target the pest species, this can consequently help farmers control pests. For example, the presence of predatory natural enemies of the invasive grain aphid (*Rhopalosiphum padi*) led to a 23% yield increase in Sweden (Östman et al., 2003). Therefore, natural enemies have the potential to reduce biochemical inputs (e.g., lower insecticide use), improve yields, prevent losses of non-target

species and lower carbon dioxide emissions (Zhang and Swinton, 2009; Heimpel et al., 2013; Jepson et al., 2014; Andersson and Isgren, 2021).

Invasive species in new territories often have fewer natural enemies than in their native range. The conifer seed bug (*Leptoglossus occidentalis*) has on average 6% of its eggs parasitised in its invaded region (Southern France) whereas in its native region (North America) around a third of its eggs are parasitised (Lesieur and Farinha, 2021). This lower threat from natural enemies is known as the *enemy release hypothesis*, that theorizes that invasive species have fewer natural enemies in a new environment as they have moved out of the range of the old natural enemies and native, potential enemies are not yet familiar with the invasive species (Cornell and Hawkins, 1993; Torchin and Mitchell, 2004). However, not all invasive species appear to escape natural enemies, the light brown apple moth (*Epiphyas postvittana*) was exposed to similar parasitism rates and species richness of natural enemies in its new environment (California) as it was in its native range (south-eastern Australia) (Bürgi and Mills, 2014).

The range, species richness and prevalence of natural enemies can vary due to many factors. The quality of the environment is a key factor, with crop richness (i.e., the number of different crops in the area) and habitat diversity (e.g., woodland, field margins, non-crop areas) linked to increased numbers of natural enemies (Shackelford et al., 2013; Ramsden et al., 2015; Maisonhaute et al., 2017). As well as physical characteristics of an environment affecting natural enemies, the presence of entomopathogenic fungi and NPV are influenced by rainfall, humidity and temperature highlighting an important role of the climate too (Fuxa and Richter, 2001; Alyokhin et al., 2011; Hajek and Tobin, 2011). Therefore, due to the many factors affecting the abundance and species richness of natural enemies there is often very little known about the natural enemies that target invasive species during the early stages of invasion.

Considering the many benefits of natural enemies, it is important to identify natural enemies in the new regions of invasive pests. This means that the effects of natural enemies on population dynamics and their potential benefits for pest management can be better understood. In Africa, natural enemies are often present against invasive pest species, and can be key in successful IPM programmes. There are three key predators and an egg parasitoid which target the invasive tomato leaf miner (*Tuta absoluta*) in Egypt, and seven parasitoids and three predators which target the invasive fall armyworm in Ghana (Koffi et al., 2020; Hassan et al., 2021). Understanding the prevalence of natural enemies in Africa

can have important consequences for farmers, particularly small holders, that often find insecticides prohibitively costly and/or inaccessible so rely on indigenous ecological resources (Grzywacz et al., 2014).

Overall biocontrol, both from natural enemies and released biocontrol agents, can play an important role in the control of invasive crop pests. Furthermore, biocontrol can offer a more sustainable approach than insecticides meaning its popularity is increasing as integrated pest management becomes more common. It is important to understand natural enemies present in an invasive species new range, this can have important consequences for both natural control and the potential for commercial biocontrol products to be approved. Considering the many benefits of biocontrol over chemical control this is a key area to consider when investigating invasive crop pests.

1.3 Migration and studying flight in insects

Migration in insects is defined as the "persistent and straightened-out movement effected by the animal's own locomotory exertions or by its active embarkation on a vehicle, it depends on some temporary inhibition of station-keeping responses, but promotes their eventual disinhibition and recurrence" (Kennedy, 1985). Unlike other types of insect movement (e.g., foraging), the undistracted movement of migration does not initially cease when suitable resources are found. This cease response is suppressed to favour persistent and straightened out movement, thus enabling longer distances to be covered (Kennedy, 1985; Dingle, 2014). To achieve this, behavioural, morphological, and physiological adaptations are all required; combined, these adaptations are collectively referred to as the *migratory syndrome*, and different components of the migratory syndrome are present in different species (Dingle, 2014).

Under some definitions of migration, many insect migrations would be classified as dispersive movements as they do not complete a round-trip between regular breeding and non-breeding areas. However, when classified in more behavioural terms as a straightened-out, persistent movement, many insects show migratory behaviour (Dingle, 2014). In insects, migration is often differentiated from dispersal if it is a repeated directional movement that is cyclical in nature, meaning that it occurs each year in the same direction (Holland et al., 2006). Some species have evolved partial migration, where some individuals migrate whilst others form resident populations, and this mechanism is found throughout the insect kingdom including in Diptera, Lepidoptera, Odonata, Hemiptera and Orthoptera (Menz et al., 2019). This approach can lead to physical differences between migratory and

resident individuals and a well-studied example of this is the two morphs of the sand cricket (*Gryllus firmus*); large winged (macropterous) and small winged (micropterous). For each morph there is a trade-off between flight capability, immunity and reproduction, in that large winged individuals have smaller ovaries, reduced immunity (e.g., reduced nodulation, age-related immunosenescence) and higher respiratory metabolism compared to the short winged morph, resulting in stronger flight ability, but at the cost of reduced immunity and lower reproductive output (Zera and Mole, 1994; Crnokrak and Roff, 2002; King, 2011).

Insect migrations that rely on flight can take place at low or high altitude. Low altitude flights occur within the flight boundary layer where insect flight speed can exceed wind speed, giving insects full control over migration direction and duration (Chapman et al., 2015). Though rare in Lepidoptera, this type of migration has been observed in the moth *Urania fulgens* in Panama (Srygley and Dudley, 2008). High altitude migratory flights take place in many Lepidoptera species. These migratory flights utilise the wind to assist long-distance, rapid flight, and insects are still capable of orientating these flights to travel in a preferred direction (Chapman et al., 2015). This migratory flight behaviour has been extensively studied in the African armyworm that uses prevailing winds each season to migrate across east Africa (Rose et al., 1985; Rose et al., 2000).

Insect life cycles are often too short for individuals to make round-trip migrations. However, inter-generational round trip migrations do occur in some species (Holland et al., 2006), as seen in the successive generations of the monarch butterfly (*Danaus plexippus*) that continue migrating north to repopulate after overwintering in Mexico (Holland et al., 2006; Flockhart et al., 2013). Similarly, the oriental armyworm (*Mythimna separata*) undertakes a multi-generation round-trip migration between southern and northern China each year (Hai-Xu et al., 2017). Alternatively, one-way migrations can occur, with genetic analysis revealing an asymmetric haplotype distribution in fall armyworm populations suggesting that return migrations do not occur after they move north from their overwintering grounds in southern Florida and southern Texas (Nagoshi and Meagher, 2008).

Flight is very energy demanding in insects, with flight alone resulting in a 50 to 100-fold increase in metabolic rate compared to rest. This rapid metabolism is powered by a range of different flight fuels including carbohydrates, lipids and proline (Beenakkers et al., 1984). However, some species adapted to undergo long-distance migratory flights have evolved to maintain lower metabolic rates so flight is more energy-efficient thus enabling

greater distances to be flown. This mechanism has been found to occur in the fall armyworm and Monarch butterfly (Nayar and Van Handel, 1971; Schroeder et al., 2020).

One way to overcome the energy costs of migration is that some insects will migrate initially and not start reproductive development or behaviour until they have landed; this is known as the oogenesis-flight syndrome (Johnson, 1969; Lorenz, 2007). This has been recorded in many species including two-spotted crickets (*Gryllus bimaculatus*), larger ground beetles (*Carabus granulatus* and *Calosoma auropunctatum*) and the boll weevil (*Anthonomus grandis*) (Johnson, 1969; Rankin et al., 1994; Matalin, 2003; Lorenz, 2007). Following flight, reproductive development often comes at the cost of reduced movement, and can follow the histolysation (i.e., breakdown) of flight muscles as a trade-off to contribute to the high energy demands of reproduction (Rankin et al., 1994; Johnson, 1969; Matalin, 2003; Lorenz, 2007). There is flexibility in how oogenesis-flight syndrome occurs in Lepidoptera. The beet webworm (*Loxostege sticticalis*) significantly reduces its flight performance after the onset of oviposition. However, flight ability in the beet webworm was not eliminated following oviposition and all females continued to fly during the oviposition period in order to deposit their eggs over a range of about 7-8km, as opposed to the initial migratory flight of 725 – 1117km (Cheng et al., 2016b). In other species, such as the African armyworm, there is evidence that the oogenesis-flight syndrome can be environmentally determined, with reproduction only delayed if sufficient food is not available (Gunn et al., 1989).

The oogenesis-flight syndrome does not occur in all migratory insect species, or even in all individuals of the same species. In two moth species, oriental armyworm and beet armyworm, there is no evidence that reproductive development was suppressed whilst migration was taking place, with individuals capable of reproducing whilst migrating (Zhao et al., 2009; Jiang et al., 2010 respectively). However, this might also be due to environmental circumstances (as observed in the African armyworm) or population differences because the oogenesis flight syndrome has been observed in some populations of oriental armyworm, with females not mating before, or during, migratory flights (Wang and Zhang, 2001; Jiang et al., 2011).

In contrast to the oogenesis-flight syndrome, some insects will favour either reproduction or flight capabilities, and this ability can change within species based on the environmental or biological circumstances. In oriental armyworm that have been exposed to migration inducing temperatures there is reduced egg production and shorter preoviposition

periods in females, and reduced response time to female sex pheromones in males (Jiang et al., 2011). Thus for some insects, if migration is triggered then reproductive efforts can be reduced in the migratory generation.

Another alternative strategy to trade-offs and oogenesis-flight syndrome has evolved in the spruce budworm (*Choristoneura fumiferana*) which is an inter-reproductive migrant, thus laying some eggs where they emerge, before migrating potentially several hundreds of kilometres and laying more eggs (Rhainds and Kettela, 2013). The oviposition threshold for this species was 50% of their eggs had to be laid before flight was possible, thus reducing the heavy weight of mature eggs to aid flight (Rhainds and Kettela, 2013). Whilst much of the research focuses on female reproductive ability in relation to migration, males also experience trade-offs, for example, male sand crickets with higher flight capabilities have lower fecundity in terms of smaller testes and shorter mating calls (Crnokrak and Roff, 1995; Crnokrak and Roff, 1998; Crnokrak and Roff, 2002).

These evolutionary changes and trade-offs between flight and reproduction that enable long-distance flight to occur highlight the importance of long-distance, migratory flights in some insect species. Understanding these costs in both males and females is important as much of work to date has only focussed on the reproductive trade-offs experienced by female flyers. By increasing knowledge of the costs of flight it will help to understand the spread of invasive species, many of which rely on long-distance flight to reach favourable environments for reproduction and survival.

1.3.1 Drivers of migration in insects

There can be many different factors that drive insect migration, and the relevance of those factors can vary by species. The drivers of migration differ in obligate and facultative migrants, with obligate migrants always undergoing migration whereas as facultative migrants may not migrate (e.g., resident individuals or not in every generation). Here, some drivers of migration are discussed in migrants which can be both facultative, such as the cotton bollworm, or obligatory migrants, such as the European corn borer (*Ostrinia nubilalis*) (Dorhout et al., 2008; Jyothi et al., 2021).

Migration can be initiated as a result of environmental conditions. Common green darners (*Anax junius*) migrate south if nocturnal temperatures are lower than a threshold for two consecutive days (Wikelski et al., 2006). Temperature triggers can occur in multiple development stages, and do so in both the larval and adult stage in the oriental armyworm, in which larvae are triggered to migrate at around 27°C and resident adults become migrants

if they are exposed to cold temperatures of around 5°C (Jiang et al., 2011). Furthermore, there are ideal temperature ranges in which flight takes place, with optimum flight occurring between temperatures of 17-22°C at ground-level for the oriental armyworm, with the distance flown significantly reducing below or above this range. Consequently, long distance migratory flight is limited by environmental temperature gradients, adding another level of environmental dependency to migration (Jiang et al., 2011). Seasonal, environmental cues can also determine the direction of migration in Lepidoptera, with the silver Y moth (*Autographa gamma*) orienting flight headings to migrate north in Spring as temperatures warm and then back towards the south in Autumn as temperatures cool (Chapman et al., 2008).

Photoperiodic cues can be important triggers for migration and are often more reliable than temperature alone. This adaptation is used in many insect species, such as the oriental armyworm and silver Y moth (*Autographa gamma)*, with shorter photoperiods triggering migration, assumedly a cue for the onset of winter, when temperatures drop below those in which they can survive (Han and Gatehouse, 1991; Hill and Gatehouse, 2009; Jiang et al., 2011). Additionally, photoperiod can lead to important changes in migratory species' ability to handle long distance flights. In the fall armyworm shorter photoperiods led to longer flights with reduced mass loss compared to longer photoperiods, suggesting they have a physiological response to shorter photoperiods that affects how they use and store energy during long distance flights (He et al., 2021).

Population density is a well-documented trigger for migration in insects, and one that can work independently, or in combination with, other environmental cues. Overcrowded African armyworm larvae produce moths that are better adapted to migration, with more glycerides to be used as fuel and an increase in female flight performance (Gunn and Gatehouse, 1987; Woodrow et al., 1987). By contrast, in the oriental armyworm, migration is triggered by intermediary population densities, with one study showing that flight performance was greatest when 10 larvae were crowded in a 850ml jar, compared to 1, 20, 30 and 40 larvae per jar (Luo et al., 1995). However, population density is not a driver of migration in all insect species. In the fall armyworm, there is no effect of larval density on lipid reserves and wing-loading, both of which are indicators of migratory flight propensity (Ferguson et al., 1997). Whilst these experiments provide evidence of population density triggering migration in the lab, field evidence for population density driven migration comes from large outbreaks of migrating insects. This has been observed in outbreaks of African

armyworm when convergent winds lead to large concentrations of dispersing migrants (Rose et al., 1985; Rose et al., 2000).

Finally, environment quality can be an imperative trigger for migration. In the oriental armyworm, periodic starvation of larvae led to more migrants within the population suggesting that they were trying to escape food stress to maximise survival of their future offspring (Jiang et al., 2011). However, this trigger has not been found across all Lepidoptera as migratory traits, such as preoviposition period, were not affected by starvation in larvae of the rice leafroller (*Cnaphalocrocis medinalis*) (Yang et al., 2014). Furthermore, the impact of starvation can vary across life stages as migrant adult oriental armyworm exposed to starvation within 24h following eclosion switched to being resident (Jiang et al., 2011).

In summary, there are many factors that can trigger migration in insects. Migration can be triggered by a combination of environmental cues which signal seasonal changes or with changing quality of an environment. Whilst the cues for migration vary across species, the underlying assumption is that these adaptations improve reproductive success by maximising offspring survival in the next generation, enabling a species to reproduce year-round and escape unfavourable environmental conditions (Chapman et al., 2015).

1.3.2 Studying insect flight in the laboratory

Many insects, including Lepidopteran species, migrate through self-powered flight (Chapman, 2012; Chapman et al., 2015). Given their small size, it is difficult to track and study insect migration in the wild, although advances in methods such as entomological radars, genetics and stable isotopes are making this easier (Nagoshi et al., 2010; Chapman, 2012; Hobson et al., 2021). For this reason, a large portion of our knowledge stems from laboratory experiments, which have been used to study multiple aspects of insect flight including behaviour, environmental cues, physiological conditions, and how host-pathogen interactions can change flight capacity (Pearson, 2016; Minter et al., 2018). Though it cannot fully represent wild, free flight in insects, studying flight in the laboratory can give an insight into many of the aspects that affect insect flight, including migratory triggers, flight speed, flight duration and distances covered (Chapman et al., 2015; Minter et al., 2018).

The evolution of insects reared in the laboratory for migration studies must be considered as there is no selection pressure favouring flight or migration. Consequently, over time non-migratory individuals can become dominant in the population even when the captive population is specifically reared for flight (Masaki and Seno, 1990). In some organisms this can be partly accounted for by controlling the rearing environment;

incubation at 28°C produces long-winged morphs and 25°C produces short-winged morphs for the sand cricket, however, over time the long-winged morph adapted to the laboratory with an increase in fecundity and reduced flight capacity (Roff and Fairbairn, 2007a). Similarly, in Lepidoptera, important migratory traits (e.g., wing elongation) can rapidly decline within 12 months of captivity even when reared in conditions that favour migration (Davis et al., 2020). To overcome this challenge, it is important to carry out flight experiments as soon as possible after collecting insects from the field to ensure they are as representative of wild insects as possible.

To study insect flight in the laboratory there are three mechanisms; tethered-flight mills, wind tunnels and free-flight chambers (Vogel, 1966; Blackmer et al., 2004; Qin et al., 2018; Minter et al., 2018). Whilst each of these methods has its advantages and disadvantages, in this literature review we focus on tethered-flight mills as this is how flight in insects was studied in this thesis.

Using tethered-flight mills for flight experiments involves attaching an insect to a rotating pivot, enabling the insect to fly freely in a circle around the central point (Dingle, 2014; Minter et al., 2018). There are different forms of tethered- flight mills but each follows the same basic principle of a rotating arm and a pivot, with a sensor detector to record the speed and distance flown (Dorhout et al., 2008; Taylor, 2010; Jones et al., 2015; Ribak et al., 2017; Minter et al., 2018). Recently, flight mills using small tethers with minimal friction have been developed which could simulate more natural flight due to the insect being less restricted (Fig. 1.1, Jones et al., 2015; Minter et al., 2018). The flight mills used for experiments in this thesis are the same minimal friction models as those shown in Fig. 1.1.

One of the disadvantages of studying insect flight using tethered flight mills is that flight behaviour is not as natural as free flight. Flight mill resistance is greater than air resistance meaning higher energy expenditure is needed during flight on a flight mill (Ribak et al., 2017). It has previously been shown that flight speed on a flight mill can be up to three times slower than free flight in the emerald ash borer (*Agrilus planipennis*), meaning that shorter distances are flown and speeds do not reflect those observed in more natural environments (Taylor, 2010). Similarly, reduced speed, distance and duration of flight was observed when the African armyworm, fall armyworm and cotton bollworm were flown on flight mills, however, the number of flights increased which showed an underlying drive to fly, similar to the persistent movement associated with migration (Pearson, 2016). Therefore, though the distances and speeds recorded on flight mills may not reflect natural

behaviour, these experiments are a useful, reliable mechanism for studying migratory behaviour of insects in the lab environment (Pearson, 2016).

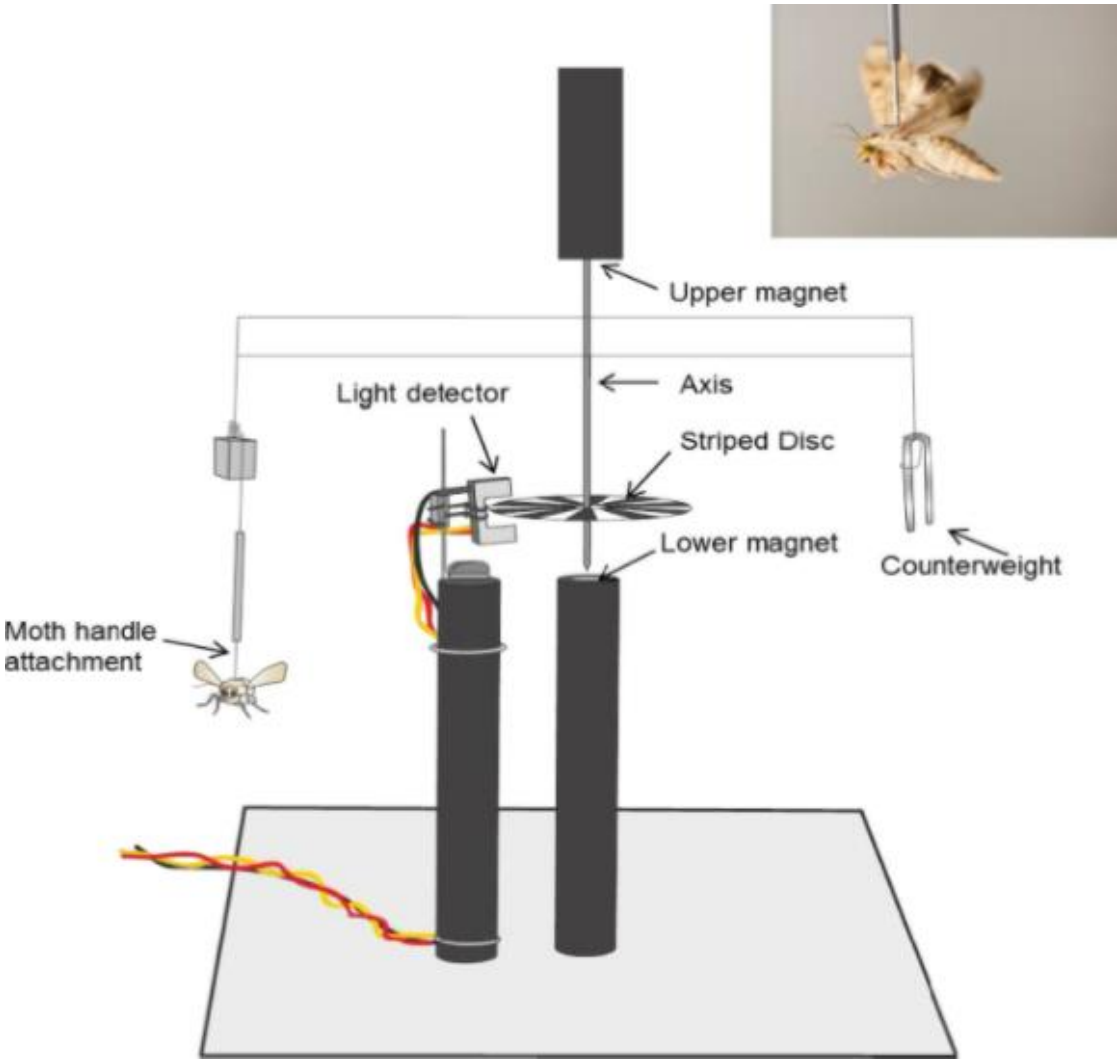


Figure 1.1 Flight mill used to study Lepidoptera flight. The image (inset) shows the cotton bollworm (Helicoverpa armigera) attached onto a flight mill and the flight mill system used to enable a smaller tether to be used. Figure taken from Jones et al., (2015).

1.4 The interaction between disease, immunity, and migration

The interaction between disease, immunity and migration is complex. Migratory species may be more at risk of parasite infection, with long-distance travel to new environments and population mixing increasing a susceptible individuals' likelihood of exposure to parasites and pathogens (Chapman et al., 2015; Koprivnikar and Leung, 2015). On the other hand, migration can reduce a species risk of infection by lowering pathogen prevalence in migratory populations as infected individuals are less likely to successfully complete migratory flights due to the energy demands of both flight and immunity (Chapman et al., 2015).

The spread of disease via migration in insects is typically associated with vectors spreading diseases that affect other species (Lounibos, 2002). This can lead to diseases such as onchocerciasis in humans being hard to eradicate as *Simulium* blackfly migrate leading to the spread of the causal parasite *Onchocerca volvulus* (Takaoka, 2015; Koala et al., 2019). There is some evidence from other taxa (e.g., birds and mammals), that migratory behaviour can also increase an individual's exposure to pathogens and increase the spread of diseases that affect the migratory individuals directly (Altizer et al., 2011). One mechanism through which this occurs is migratory individuals gathering at hotspots for disease transmission on long-distance migrations, predominantly at stopover sites where crowding happens (Altizer et al., 2011; Fritzsche McKay and Hoye, 2016). This increased pathogen exposure leads to interactions with immunity as it increases the energy demands of the immune system as more will be invested into defence systems (Koprivnikar and Leung, 2015; Chapman et al., 2015). These interactions can affect the evolution of both host and pathogen, for example, covert infections by baculoviruses may have evolved to aid the persistence and dispersal of the pathogen in migratory populations of insects (Vilaplana et al., 2010; Williams et al., 2017). However, to date, evidence suggests that migratory insects are more likely to have lower pathogen loads compared to non-migratory insects (Altizer et al., 2000; Simmons and Rogers, 1991).

The interaction between migration and disease is important as pathogens are likely to be one of the drivers behind the evolution of migration (Altizer et al., 2011; Chapman et al., 2015; Johns and Shaw, 2016). This can be through several mechanisms that all interact with each other; *migratory escape, migratory culling, non-selective migratory mortality,* and *migratory recovery*. Migratory escape occurs when migrating individuals escape environments with high pathogen abundance. This is necessary for some species as parasite prevalence can rise when population size is high, especially during breeding seasons when populations increase (Bartel et al., 2011). Another mechanism is via migratory culling in which heavily burdened individuals are removed from the population due to being unable to survive the high energy demands of migration, thus reducing transmission of the pathogen and removing susceptible individuals from the gene pool (Hall et al., 2014). Additionally, non-selective migratory mortality reduces disease prevalence through lowering population density and reducing transmission of pathogens from infected individuals to susceptible individuals (Johns and Shaw, 2016). Finally, migratory recovery is when individuals lose their parasites and recover during migration, this can occur because parasites are unable to

survive environmental differences or physiological changes in their hosts triggered by migration (Shaw and Binning, 2016; Shaw and Binning, 2020).

Furthermore, theoretical modelling has shown that the level of tolerance to disease can play a crucial role in whether migration occurs (Kim and Shaw, 2021). If tolerance to a disease is low then disease-induced mortality is high and fewer individuals will migrate, whereas if tolerance is high most infected individuals survive and more migrate as the benefits of migration outweigh the costs (Kim and Shaw, 2021). However, increased migration may only occur to a certain point if migration is driven by a drive to escape disease (i.e., migratory escape or migratory recovery) because with high tolerance then the benefits of migration decrease as survival remains high regardless of infection (Kim and Shaw, 2021).

In addition, the interactions between migration and disease can be influenced by many factors, including the environment. Behavioural changes driven by the environment occur in Mormon crickets (*Anabrus simplex*) that adapt their diet during the migratory period to aid immune function, at the cost of reduced speed during the migration (Srygley and Lorch, 2011). Crickets collected from the migratory region in Nevada selected carbohydrate-rich diets over protein-rich ones, which resulted in reduced migratory speed but increased immune responses via an enhanced ability to encapsulate foreign particles and lyse bacteria (Srygley and Lorch, 2011). This reduces the risk of disease when encountering novel pathogens during migration. However, this response was not observed throughout all populations of Mormon crickets. Populations from Utah preferred a protein-rich diet that increased phenoloxidase activity compared to those fed a carbohydrate-rich diet (Srygley et al., 2009). These differences in diet response in the two populations suggests that there are local adaptations to macronutrient differences to ensure that immune responses are maintained.

The interaction between migration and disease varies between species, pathogen virulence and the type of migration. It is likely to involve trade-offs between the physiological costs associated with migration (e.g. changes in morphology and metabolism) and susceptibility to disease (e.g. disease resistance, immune responses) (Chapman et al., 2015). The extent to which the ability of an individual to migrate is affected by the pathogen might occur in multiple ways; a triggered response, where the exposure to a pathogen initially increases migratory behaviour before a subsequent decline, a linear or non-linear cost infection, no cost (negligible) or as a step function, where the migrant can tolerate infection up to a certain threshold (Fig. 1.2a). The immune response (i.e., susceptibility to

disease) may change with migratory effort (i.e., the amount of energy invested in migratory traits). Again, the relationship can occur as a step function, a linear cost, a non-linear cost or have no cost (negligible), with migratory culling occurring when the susceptibility to disease exceeds the individual's ability to invest in migratory effort, resulting in infected individuals being removed from the migratory population (Fig. 1.2b).

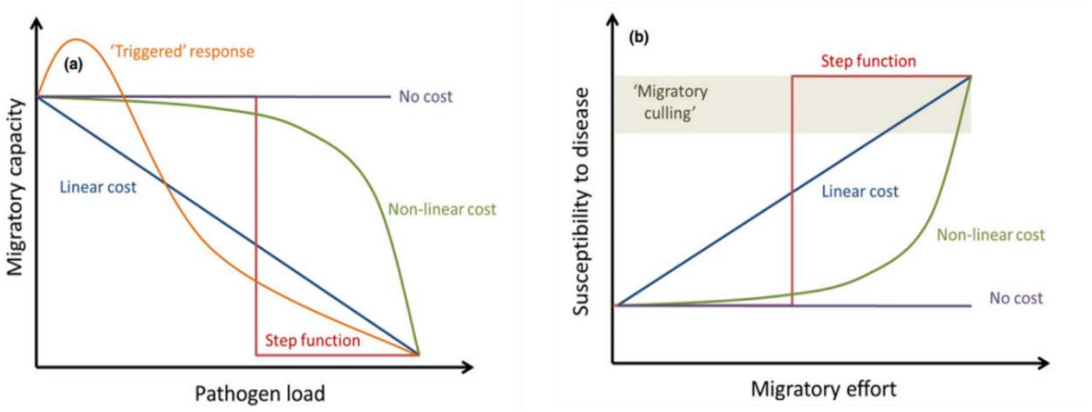


Figure 1.2 The interactions between migration and disease are complex and vary between species. a) Migratory capacity is reduced by pathogen load. B) Susceptibility to disease is increased by migratory effort. Figure taken from Chapman *et al.,* 2015.

The most well-studied example of the disease and migration interaction in insects is that of monarch butterflies and the obligate protozoan parasite *Ophryocystis elektroscirrha*. In North America, monarchs are either migratory or resident and the parasite *O. elektroscirrha* infects them throughout their geographical range, but prevalence varies between migratory and resident populations. Parasite prevalence is linked to migratory ability, with infected monarch butterflies flying shorter distances, flying slower and losing proportionally more body mass per kilometre flown compared to non-infected butterflies (Bradley and Altizer, 2005). Thus, there is a clear cost associated with infection, with several studies showing that migratory capacity decreases as pathogen prevalence increases (Altizer et al., 2000; Bradley and Altizer, 2005; Bartel et al., 2011). It is estimated that it would take infected monarch butterflies an average of 6 days longer to reach the overwintering site, and some may be unable to survive migration (Bradley and Altizer, 2005). Further supporting the theory of migratory escape, or migratory culling, in this species parasite prevalence declines during the migratory journey, resulting in lower levels of parasite prevalence in migratory monarch butterfly populations, with only 8% of migrants heavily infected compared to 70% of the resident population (Altizer et al., 2000; Bartel et al., 2011). Combined, these studies provide strong evidence that the high energy demands of migration can result in heavily infected individuals being removed from the population, thus reducing transmission of the parasite during migration and overwintering. Though less studied, this relationship has been

observed in other Lepidoptera species. Early arriving migrants of the fall armyworm were not infected by a nematode (*Noctuidonema guyanense*), or had very few nematodes, compared to later migrants, which suggests that those infected may be less able to undergo long distance flights (Simmons and Rogers, 1991).

Another factor that may influence the interaction between immunity and flight is sex, as there is evidence that males and females may respond differently to disease and flight. In the fall armyworm, infection with *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV) led to different migration strategies between males and females (Pearson, 2016). It was found that females appear to undergo migratory escape, with infection shortening development time with no change to flight capacity. In contrast, males underwent a form of migratory culling due to declines in flight capacity and no change in developmental time which led to increases in pathogen abundance (Pearson, 2016). Bateman's principle could explain differential investment in immunity between the sexes, due to females increasing fitness (i.e., the number of offspring) through longevity whereas males increase fitness through mating success (Bateman, 1948).

Overall, there is a very complex interaction between migration and disease, and this has been shown to be a strong selective pressure which could theoretically act as a driver of evolution in a species. Understanding this relationship in more detail is vital to determine what role diseases may have on the spread of invasive species, and what consequences biocontrol could have on migratory pests that are exposed to sublethal levels of biological control agents.

1.4.1 Host-pathogen ecology of baculoviruses in Insects

Baculoviruses are DNA viruses that have been identified in many insects, and are particularly widespread in Lepidoptera and Hymenoptera (Cory and Myers, 2003; Goulson, 2003). There are two types of baculovirus, nucleopolyhedroviruses (NPV) which are composed of multiple virions and granuloviruses (GV) which contain a single virion (Cory and Myers, 2003). Baculoviruses are generally host-specific and are only able to infect one host, however, some species can infect a variety of host species, and these are typically those associated with polyphagous pest species (Goulson, 2003; Simon et al., 2004).

Both NPV and GV are infectious to larval stages of insects, and the disease is transmitted horizontally between hosts as proteinaceous structures called occlusion bodies (OB) (Cory and Myers, 2003). Horizontal transmission occurs when larvae are infected with OB in the environment, such as through the consumption of contaminated foliage. OB

contain virions and are broken down in the alkaline midgut to release the virions which then enter the hosts cells where they rapidly replicate using the host nucleus (Cory and Myers, 2003; Thiem and Cheng, 2009). Additionally, baculoviruses can be transmitted vertically between parents to offspring through the virus reducing virulence to cause covert infections (i.e., no visible signs of disease) (Williams et al., 2017). Vertical transmission can occur in two ways; transovarial (within eggs) or transovum (on egg surface during oviposition) (Cory and Myers, 2003). Due to the differences in transmission, the virulence of vertically transmitted baculoviruses is lower than that of horizontally transmitted viruses (Cabodevilla et al., 2011). This reduced virulence and pathogenicity enables the spread and persistence of the baculovirus within populations as infected individuals can complete long-distance flights and successfully reproduce (Williams et al., 2017). This covert strategy means that *Spodoptera exempta nucleopolyhedrovirus* (SpexNPV) can persist at high levels in populations of migratory African armyworm (*Spodoptera exempta*), with up 78-100% of some populations being PCR positive for SpexNPV (Vilaplana et al., 2010). Vertical transmission is more likely to occur at low population densities when opportunities for horizontal transmission are reduced, this ensures the virus can persist within populations, with virus infection significantly more prevalent in African armyworm eggs when the parental generation had been reared in solitary conditions compared to those that had been reared in gregarious conditions (Vilaplana et al., 2008).

Whilst it can be beneficial for baculoviruses to persist in populations by causing covert infections, they can be triggered to cause overt infections that lead to disease outbreaks within populations (Williams et al., 2017). There are many proposed triggers that cause this switch, such as environmental stresses (e.g., changes in temperature or humidity) and reduced food availability that could adversely affect host survival (Williams et al., 2017). Alternatively, infection by a second pathogen can trigger ia covert infection to activate, with *Spodoptera litura nucleopolyhedrovirus* switching from latent to active infection in *Spodoptera litura* following cross-inoculation with *Mythimna separata nucleopolyhedrovirus* (Kouassi et al., 2009).

The pathogenicity and virulence of baculoviruses can vary significantly between isolates (Cory and Myers, 2003). This variation is maintained via a variety of mechanisms, such as differential selection (i.e., different traits being beneficial in different environments), trade-offs and interspecific competition (Cory and Myers, 2003). In Lepidopteran baculoviruses a trade-off occurs due to the production of OB being negatively correlated with speed of kill, meaning that the virus cannot spread to a new host horizontally until it

kills its current host, but if it kills too soon it will have produced fewer OB so will have lower transmissibility (Redman et al., 2016).

Determining the pathogenicity and virulence of various baculovirus species and strains within crop pests is important as baculoviruses are beneficial as biopesticides due to their high host-specificity and efficiency (Lacey et al., 2015a; Bateman et al., 2021; Behle and Popham, 2012; Landwehr, 2021; Fuxa, 2004). Baculovirus strains are often isolated and compared in laboratory experiments to determine their suitability for potential use as biocontrol agents based on virulence and pathogenicity, for example eight isolates of *Helicoverpa armigera NPV* were compared to determine their potential as a biocontrol agent for *Helicoverpa armigera* in Spain which identified one as most suitable due it having the fastest speed of skill (Arrizubieta et al., 2014).

Insects have developed numerous mechanisms of resistance to protect against baculoviruses. Resistance has been shown to vary considerably between species and even between populations of the same species. African armyworm reared at high population density haver greater resistance against the baculovirus SpexNPV compared to those reared at lower densities (Reeson et al., 1998; Wilson and Reeson Andrew, 2002). A potential mechanism behind this population density dependent resistance could be variation in melanisation, with gregarious populations having higher melanin levels which is associated with increased immunity activity (e.g., increased phenoloxidase activity) (Reeson et al., 1998; Wilson et al., 2001). Alternatively, other Lepidopteran species have evolved mechanisms to prevent baculovirus replication, as observed in codling moth (*Cydia pomonella*) populations that were resistance to the biocontrol agent *Cydia pomonella granulovirus* (CpGV) as they were able to prevent baculovirus replication in cells once CpGV had entered them (Asser-Kaiser et al., 2011).

Insect immunity is highly complex, and the specific pathways involved in the response to baculoviruses are not confirmed. Various immune responses have been implicated in Lepidoptera response to baculoviruses, including increases in hemocytes following infection in *Helicoverpa zea (Black et al., 2022)* and *Lymantria dispar* (McNeil et al., 2010) and the JAK/STAT pathway being initiated in response to *Bombyx mori nucleopolyhedrovirus* in *Bombyx mori* (Cheng et al., 2016a). However the response may vary between species with RNA-sequence analyses showing that the Toll, Imd and JAK/STAT pathways all showed no response following infection of *Helicoverpa armigera* with *Helicoverpa armigera nucleopolyhedrovirus (Xing et al., 2017)*.

Overall, the host-pathogen ecology of baculoviruses is highly complex with many questions yet to be answered, particularly those surrounding the immune responses of insects to baculoviruses and the potential impacts of covert infections. In this thesis the focus is on improving our current understanding of covert baculovirus infections, and the ways in which these could affect the spatial dynamics of the fall armyworm through their impacts on insect populations and flight ability.

1.5 The genetics of migration

Migration occurs as an interaction between the environment, the individual and the genome (Fig. 1.3). All migrant species have a *migratory syndrome* that comprises a suite of traits that all have a genetic component (Dingle, 2014). The traits that form the migratory syndrome include morphological and physiological features, as well as a set of responses to cues that can trigger migration (Fig. 1.4). Consequently, natural selection drives migration and the establishment of the migratory syndrome through individual migrants increasing fitness by reaching more favourable environments, or alternatively by migrants being removed from the gene-pool if they are unable to survive migration (Dingle, 2014).

There is strong evidence that the genetic influence on migration is greater in females than males, and many insect species show a female-bias in the numbers of migratory individuals (Albrectsen and Nachman, 2001; He et al., 2018; Guo et al., 2019). Possible explanations for this female-bias include females investing more into migratory flight ability to increase longevity, or a drive to reach favourable habitats to lay eggs, whilst males favour a sit-and-wait strategy to maximise mating success (Albrectsen and Nachman, 2001; He et al., 2018; Guo et al., 2019). The quantitative genetics of this female bias was studied in the African armyworm*,* and female offspring showed differences in the pre-reproductive period after just one generation of selection, however, this relationship was not observed in males (Wilson and Gatehouse, 1992; 1993). Furthermore, the pre-reproductive period appeared to be largely explained by genetic differences between African armyworm populations rather than environmental variables (e.g., temperature and humidity) (Wilson and Gatehouse, 1993).

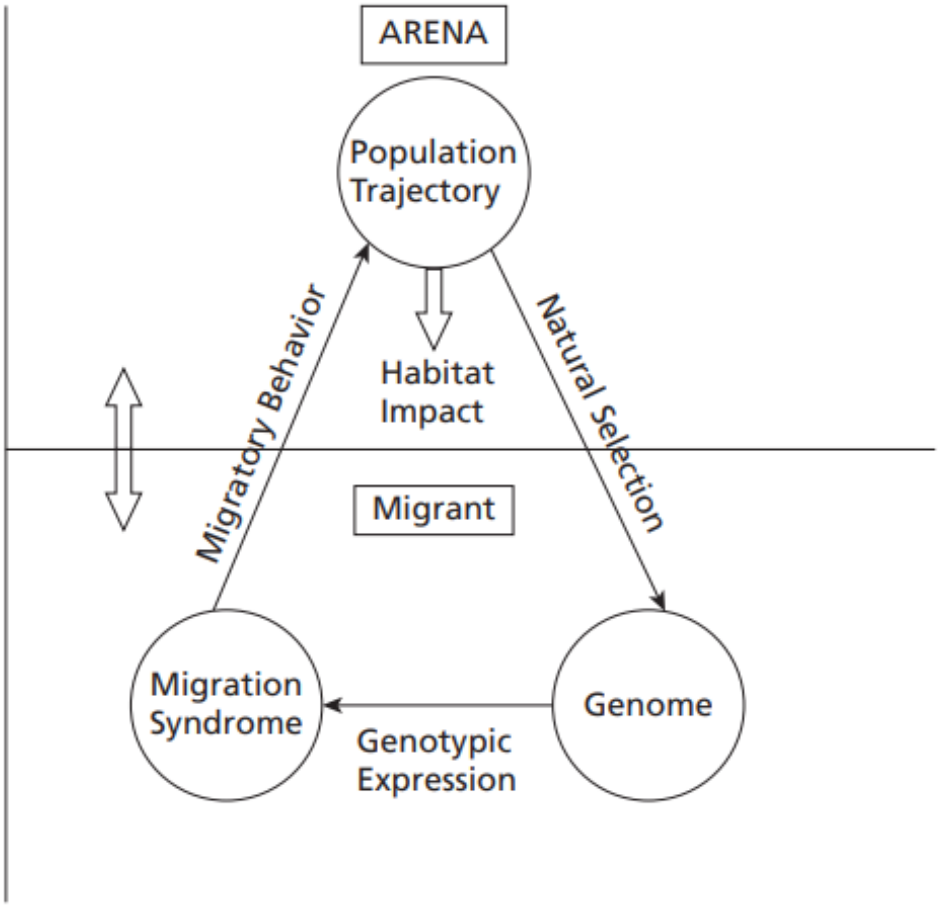


Figure 1.3 The migration system. A holistic model of migration that shows how the population trajectory (route and timing of migration), migratory syndrome (a series of traits needed for migration) and genome (underlies migratory syndrome and responses to environment) all interact. The Arena area shows the environmental component (biotic and abiotic) and the Migrant area shows the individual component. Interactions between these components are driven by natural selection and can result in different migratory behaviours for individuals e.g., varying trajectories and survival rates. Image taken from Dingle (2014).

Genetic variation is essential for natural selection, without variation in migratory traits driven by the environment then the trait will not be under selection. There is evidence that genetic variation is potentially maintained by trade-offs like those associated with flight, reproduction, and immunity. Genotypes that allocated more resources into one trait in one environment may allocate more resources to the other trait in a different environment. In conditions with excess food, large-winged crickets increased their allocation of reproductive resources to a greater extent than they did for flight capability (King, 2011). This highlights that there is a strong genotype-by-environment interaction underlying the trade-off between acquisition and allocation, and consequently there is likely to be high genetic variation in the migratory trait (King, 2011).

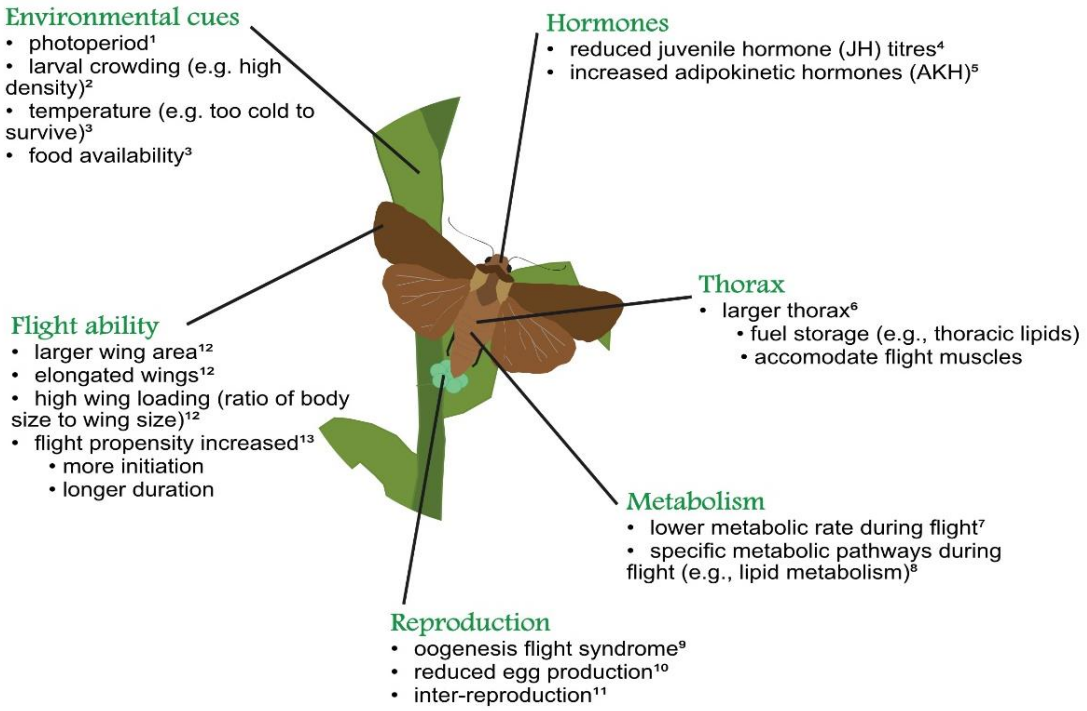


Figure 1.4 Migratory syndrome in Lepidoptera. The migratory syndrome is a series of morphological, physiological and responses to the environment that mean an individual can migrate. This figure shows some of the main traits of the migratory syndrome in Lepidoptera, and superscript numbers provide a reference for evidence of this in migratory Lepidoptera. 1: (He et al., 2021), 2: (Gunn and Gatehouse, 1987), 3: (Jiang et al., 2011), 4: (Zhu et al., 2008a), 5: (Marco et al., 2020), 6: (Berwaerts et al., 2002), 7: (Nayar and Van Handel, 1971), 8: (Jones et al., 2015), 9: (Cheng et al., 2016b), 10: (Gunn et al., 1989), 11: (Rhainds and Kettela, 2013), 12: (Freedman and Dingle, 2018), 13: (Dällenbach et al., 2018).

1.5.1 Identifying the genes driving migratory behaviour

Migration is composed of multiple traits, known collectively as the migratory syndrome (Fig. 1.4). Therefore, no individual genes or traits drive migration alone. It is the interactions between genes influencing different traits, and the interactions of these traits with the environment that lead to migration (Roff and Fairbairn, 2007b; Dingle, 2014). However, advances in genomics and transcriptomics have allowed us to pinpoint some of the key genes and pathways involved in long distance flights in insects (Zhu et al., 2008a; Zhan et al., 2011; Jones et al., 2015).

Genetic analysis comparing the same population during the migratory season with the non-migratory season enables specific genes involved in migration to be identified. Using these methods, a number of key migratory genes involving sensory input, hormone regulation and orientation have been identified in the monarch butterfly (Zhan et al., 2011). Similarly, in the cotton bollworm, transcriptomic comparisons between adult moths displaying a continuum of flight capabilities revealed specific genes related to long-distance flight involved in energy production, fatty acid break down and mobilization, as well as genes

involved in protecting against oxidative damage (Jones et al., 2015). Some genes were also found to be downregulated, including those involved in fatty acid synthesis and allatotropin receptors which are involved in juvenile hormone (JH) synthesis (Jones et al., 2015). Reduced JH levels have been associated with inducing reproductive diapause and increasing longevity in migratory monarch butterflies (Zhu et al., 2008a). A summary of the key genes that have been found to be differentially expressed during flight in Lepidoptera is provided in Table S1 in Appendix A.

Gene expression profiles of migratory and non-migratory monarch butterflies further support the complexity of the migratory syndrome, with 40 individual genes identified that have a role in migratory behaviour (Zhu et al., 2009). One area where specific genes necessary for migration have been identified in the monarch butterfly are those involved the time-compensated compass (Perez et al., 1997). The time-compensated compass is critical for monarch butterfly migration directionality, meaning that in fall migrants travel from east North America to South America (Mexico). These specific genes are linked to the circadian clock, including *vrille*, *cry1* and *cry2,* and have roles in the timing of migration and in initiating, or maintaining, flight activity (Zhu et al., 2008b; Zhu et al., 2009). Further investigation into *cry1* expression in monarch butterflies has discovered a role in light-dependent magnetosensing that could help provide directional information during migrations (Wan et al., 2021). Similarly, *magnetorecepter* and *cryptochrome* genes were upregulated in migratory populations of the black cutworm (*Agrotis ilsilon*) compared to a laboratory population, and there was some evidence of directionality cues with higher levels in the population migrating southward compared to the northward migrating population (Chang et al., 2019).

Until recently, migratory genetics has been much better studied in avian migrants compared to insects. Seasonal plasticity in gene expression linked to migration has been identified in birds, such as the Swainson's thrush (*Catharus ustulatus*) in which 188 genes were differentially regulated between migratory and non-migratory birds. These were involved in a range of functions including the regulation of the thyroid hormone, cell motility, adhesion and possible morphological differences (Johnston et al., 2016). However, whilst identifying candidate genes can suggest genes that are involved in migration this must be used with caution as direct cause and effect is not established, and when investigated further, candidate genes do not always distinguish between migratory and non-migratory individuals (Lugo Ramos et al., 2017). ADCYAP1 was highlighted as a potential gene controlling the expression of migratory behaviour in birds, with the behaviour determined by

gene length (Mueller et al., 2011). However, whilst a correlation was observed between ADCYAP1 and migratory distance this was not able to distinguish between migratory and non-migratory individuals (Lugo Ramos et al., 2017). Furthermore, the role of ADCYAP1 in migration in birds is yet to be identified, with previous work hinting at a role in migratory restlessness, however a later study looking at this in more depth found no link between ADCYAP1 gene polymorphisms and migration date (Mueller et al., 2011; Saino et al., 2015). Another candidate gene with contradictory roles in migration in birds is CLOCK, with previous work suggesting that the number of glutamine residues in the poly-Q region was linked to migration date, however, later studies found no evidence of CLOCK variation linked to migratory phenotypes (Saino et al., 2015; Lugo Ramos et al., 2017). Therefore, it is important to consider when carrying out genetic work that some of the candidate genes might appear to play a role but have a different role once investigated in further detail.

Overall, there is strong evidence that the genome and genetic variation play a large role in migration, both in triggering migratory behaviour and in the adaptations that enable an individual to undergo such high energy demanding, prolonged activity. Understanding the response to flight at the molecular level will greatly improve our understanding of some of the mechanisms behind the observed behaviours related to flight in invasive species, and could further explain key differences both between species, and within species (e.g., individual variation, sex differences).

1.5.2 Impacts of migration on population genetics

Migratory species often show little genetic structure due to the extent of genetic mixing between migratory and resident populations. In overwintering sites there are high levels of genetic mixing in species that then migrate in summer, as observed in numerous insects including in hoverflies (*Episyrphus balteatus* and *Sphaerophoria scripta*) (Raymond et al., 2013) and the small brown plant hopper (*Laodelphax striatellus*) (Zheng et al., 2016). Additionally, immigration into areas increases genetic diversity, with genetic diversity in the pygmy grasshopper positively correlated to the number of recent immigrants to an area (*Tetrix subulata*) (Tinnert et al., 2016). This genetic mixing with migratory individuals maintains high genetic diversity, which can increase the fitness of a species through higher adaptive potential and colonization abilities as they are better equipped to deal with new or changing environments (Caprio and Tabashnik, 1992; Raymond et al., 2013).

Rapid gene flow (i.e., the movement of genes between populations) is associated with invasive species, and is one of the factors that enable migrants to colonize new areas

due to the higher levels of adaptive phenotypic plasticity that it can provide (Davidson et al., 2011; Knop and Reusser, 2012). This increased phenotypic plasticity gives invasive species a competitive advantage over native species. The invasive fire ant, *Solenopsis invicta,* has higher plasticity compared to the native fire ant, *Solenopsis geminate*, meaning that the invasive fire ant outcompetes the native fire ant as it is able to acclimatise better to extreme conditions (e.g. temperature, humidity) and respond better to habitat destruction (Manfredini et al., 2019). Phenotypic plasticity can also give invasive agricultural pests an advantage, such as the wide range of detoxifying genes in the fall armyworm that enable it to take advantage of many different host plants (Silva-Brandão et al., 2017; Montezano et al., 2018).

In invasive crop pests, long-distance migration that leads to rapid gene flow can be particularly problematic for agricultural systems due to the spread of insecticide resistance genes. In the highly invasive cotton bollworm, long distance flight maintains high population diversity which has led to the rapid spread and maintenance of resistance genes, including alleles related to Bt resistance (Xiao et al., 2017; Jones et al., 2019), and pyrethroid resistance (Anderson et al., 2016).

Long-distance flights leading to increased gene flow between populations in migratory crop pests makes it particularly important to understand these movements in their new habitats. Understanding how populations are mixing, and where populations could spread to next, is vital in reducing the risk to crops. It can help farmers to plan ahead to mitigate damage, as well as be more able to manage pest outbreaks by knowing what resistances the pest might be carrying. In invasive populations, resistances can often be different to in the pests' native range due to factors such as genetic bottlenecks, new migratory routes and different selection pressures (Zhang et al., 2020; Yainna et al., 2021). Therefore, understanding genetic variation and population structure in an invasive crop pest is critical to help control the pest.

1.6 Overview of the fall armyworm (*Spodoptera frugiperda*)

Fall armyworm is a member of the Lepidoptera order, in the Noctuidae family. Its native host range is in the tropical and subtropical regions of the Americas, where it can survive winter in Florida and Texas before spreading north in early May (Nagoshi and Meagher, 2008). It is a generalist species, with hosts found in 27 plant families, including Poaceae, Brassicaceae and Solanaceae. These families include many important crops such as maize (*Zea mays*) and rice (*Oryza sativa / Oryza glaberrima*) (Jeger et al., 2017).

In early 2016, the fall armyworm was first detected in south-western Nigeria on maize, before spreading north into other countries causing warnings to be issued for a potential outbreak in June 2016 (Goergen et al., 2016). The introduction of fall armyworm to Africa was accidental, possibly due to air transport or food produce from America. Multiple genetic studies have since shown that the fall armyworm is likely to have been introduced via a single, recent event based on limited genetic diversity and the absence of genetic markers that are widely distributed in the Americas (Abrahams et al., 2017; Nagoshi et al., 2018b; Nagoshi et al., 2019b). After the initial invasion, fall armyworm continued to spread and by September 2017 it was confirmed in 28 African countries, and by 2018 it had spread to at least 44 African countries. From Africa, it continued its global spread, reaching Asia between late 2018 and early 2019, and Australasia in 2020 (CABI, 2020; Food and Agriculture Organisation of the United Nations, 2021). The current distribution of fall armyworm is shown in Fig. 1.5.

Fall armyworm has devastating effects on crops, with 8.3 - 20.6 million tonnes of maize predicted to be lost per year in Africa, which is 21-53% of the average annual production so this loss will result in food shortages (Abrahams et al., 2017). In addition, the economic burden of this loss is predicted to be $2481 million – 6187 million each year increasing economic stress and could be catastrophic to individual farmers (Abrahams et al., 2017). The devastating effects of fall armyworm on crops was highlighted in Malawi. In April 2017, the fall armyworm was confirmed as present in Malawi after being first detected in January 2017 (International Plant Protection Convention, 2017). By mid-December 2017, Malawi declared 20 districts affected by fall armyworm as disaster areas, due to thousands of hectares of fields infested which impacted on at least 133,000 families (Kalilani, 2017).

There are two strains of the fall armyworm; corn and rice. They are named after the crop host they are usually associated with, however, both strains are generalist species that can survive on other plants (Pashley, 1986). There has been a lot of confusion over whether the invasive fall armyworm in Africa are the rice or corn strain, both or a hybrid between the two. This is due to discrepancies between *mitochondrial cytochrome oxidase I* (*COIB*) and the Z-chromosome-linked *Triosephosphate isomerase* (*Tpi*) markers, with *COIB* identifying many samples as rice whereas *Tpi* identifies the same samples as corn (Nagoshi et al., 2018a; Nagoshi et al., 2019b; Nagoshi et al., 2020). Furthermore, most samples were collected on plants associated with the corn strain (e.g., maize, sorghum) thus raising suspicion of whether these are the corn strain despite the *COI* marker identifying them as the rice strain.

Other work identified the strains present in Africa and found the corn strain present in Tanzania, Sao Tome and Principe and the rice strain in Nigeria (Goergen et al., 2016; Srinivasan et al., 2018). Both Georgen *et al.,* (2018) and Srinivasan *et al.,* (2018) relied on the *COI* marker to identify the strain thus no discrepancies between the results would have been detected. However, both studies do report that in locations where the rice strain has been identified it is predominately corn-strain associated plants affected (Goergen et al., 2016; Srinivasan et al., 2018). Further research using genetic sequencing revealed that this discrepancy is most likely due to hybridisation between the rice and corn strains, occurring shortly after fall armyworm first arrived in Africa, meaning that fall armyworm in the invaded regions are a hybrid of the corn and rice strains found in its native region (Zhang et al., 2020).

Figure 1.5 The current distribution of fall armyworm (*Spodoptera frugiperda*). It is native to North and South America (pre-2016), and invasive in Africa, Asia, and Australasia. Figure is from Food and Agriculture Organisation of the United Nations (2021). It is native to North and South America, and invasive in Africa, Asia and Australasia. Figure is from Food and Agriculture Organisation of the United Nations (2021).

The rapid spread of the fall armyworm is largely caused by its strong flight ability, with individuals able to disperse around 300 miles before oviposition (Johnson, 1987). Additionally, its life-history enables rapid reproduction, with each female able to lay around 1000 eggs and in warm temperatures each generation can grow from egg to adult in 30 days (Johnson, 1987; Murúa and Virla, 2004). The fall armyworm is unable to survive freezing thus it dies out in winter in much of its native range, and persists year-round only where temperatures are above freezing. Accordingly, the furthest north it can survive year-round on the American continent is southern Florida and southern Texas where its generation time slows in winter but survival is possible (Nagoshi and Meagher, 2008; Westbrook et al., 2016). It is thought that fall armyworm outbreaks will be worse in sub-Saharan Africa and Asia as

the climate allows continuous breeding throughout the year in much of the affected countries (Abrahams et al., 2017; Zacarias, 2020).

Population genetics can help us to understand the movements of fall armyworm, both to highlight areas at risk and determine the likelihood of factors like insecticide resistance spreading. However, there is very little genetic diversity based on markers previously used to look at population structure in the native region (*COIB, TpiE4* and *TpiI4* haplotypes) meaning that it is hard to fully understand the population structure of the invasive populations of fall armyworm in both Africa and Asia (Nagoshi et al., 2010; Westbrook et al., 2016; Nagoshi et al., 2017a; Nagoshi et al., 2018a; Kasambala Donga and Meadow, 2018; Zhang et al., 2020; Nagoshi et al., 2020). This low genetic diversity in the fall armyworm in Africa is expected due to it being an invasive species so the founding population was likely composed of very few individuals, however, it makes understanding population movements much more difficult.

Natural enemies play an important role in controlling fall armyworm throughout its native regions, with many known enemies occurring naturally such as SfMNPV, and numerous parasitoids and predators (Virgen et al., 2013). A factor that could have played a role in the rapid spread of fall armyworm throughout Africa and Asia is reflected in the enemy release hypothesis, that is when an invasive species can rapidly spread due to having fewer enemies (e.g., diseases, predators, parasites) than in its native region (Cornell and Hawkins, 1993; Keane and Crawley, 2002; Roy et al., 2011). Some parasitoids of eggs, larvae and pupae have been identified in Africa, including Ghana (N = 8), Benin (N = 9), Ethiopia (N = 3), Tanzania (N = 3), Uganda (N = 13) and Kenya (N = 5) (Sisay et al., 2018; Sisay et al., 2019; Agboyi et al., 2020; Otim et al., 2021). Additionally, SfMNPV has been identified in China and Nigeria (Lei et al., 2020; Wennmann et al., 2021). However, very little is known about which microbial natural enemies are present in Africa, and this is an important area to understand further as natural enemies often play a vital role in integrated pest management programs.

In its native region, fall armyworm has evolved resistance to many pesticides making it particularly hard to control, with a wide range of mechanisms recorded which give resistance to pesticides including AChE inhibitors, sodium channel modulators and inhibitors of chitin biosynthesis (Gutiérrez-Moreno et al., 2018). The impact of fall armyworm's long-distance, migratory flights on the spread of insecticide resistance is evident in the spread of resistance to genetically modified Bt maize, which was first recorded in Puerto Rico and Brazil (Storer et al., 2010; Farias et al., 2014). Through migration of fall armyworm, Bt

resistance consequently spread throughout America, including Florida and North Carolina (Huang et al., 2014). However, limited migration occurs along the lesser Antilles between Puerto Rico and South America so it is possible that resistance to Bt maize occurred independently in multiple locations in the Americas (Nagoshi et al., 2017a). In many invasive species, resistance genes are bought with them into new habitats, but sometimes resistances can change due to bottlenecks that occur during invasions so it is important to check for resistances when a species first invades. Fall armyworm carried resistance to organophosphate and pyrethroid pesticides into China when they arrived but are susceptible to Bt maize, whereas as in parts of their native region they are resistant to Bt (Zhang et al., 2020). It is particularly important to consider insecticide resistance in invasive fall armyworm as it has been found that invasive populations (Uganda, Malawi, Benin, India and China) have greater copy numbers of P450 genes and a higher proportions of alleles that confer AChE resistance compared to native populations (Peurto Rico, Brazil and Florida) (Yainna et al., 2021). This is likely to be due to strong selection pressures enforced by the high numbers of insecticides used against fall armyworm in the invaded regions (Yainna et al., 2021).

Considering the high levels of insecticide resistance in fall armyworm, biopesticides are likely to be an important method of pest control to limit the impacts of fall armyworm in Africa. The number of biopesticides registered for use against fall armyworm has increased over time, and now includes a wide range of products such as the bacterium *Bacillus thuringiensis,* the fungal pathogen *Metarhizium anisopliae* and sex pheromones (Bateman et al., 2021). These biopesticides could help to reduce farmer reliance on chemical pesticides, if the challenges of farmer perceptions and costs are managed (Bateman et al., 2021).

Baculoviruses could be particularly important in the control of fall armyworm, with the SfMNPV having mortality rates as high as 74% in larvae when eggs were inoculated (Rios-Velasco et al., 2012). High mortality was maintained in maize field trials where larvae were collected 2 days after spraying SfMNPV and mortality rates of 60% (aqueous spray solution) and 65% (granule mix) were observed (Castillejos et al., 2002). As well as high mortality rates in fall armyworm, SfMNPV is an ideal biocontrol agent as it has very limited effects on non-target species. The chemical pesticide chlorpyrifos caused a reduction of 50-70% in natural enemies of fall armyworm whereas SfMNPV did not reduce the number of natural enemies present (Armenta et al., 2003). This detrimental effect of chemical pesticides on natural enemies has been observed in other studies, for instance, parasitism of fall armyworm larvae fell from 44% to 15% in fields on which insecticides had been sprayed (Meagher et al., 2016).

Overall, the fall armyworm is a highly invasive crop pest that causes devastating crop losses wherever it is found. Understanding fall armyworm populations, and methods of control, is vitally important to help to mitigate against crop losses, reducing both the economic impact and food shortages. This thesis aims to improve our current knowledge of fall armyworm by filling in some of the important research gaps, focusing on microbial natural enemies and populations structure in Africa, but also aims to improve understanding of the interaction between flight and disease at the molecular level to fully understand the impact of SfMNPV on fall armyworm.

1.7 Thesis summary

This thesis improves current knowledge of disease and migration in fall armyworm by using a combination of population studies, flight mill experiments and molecular analysis. It identifies microbial natural enemies present in fall armyworm in Africa for the first time and uses microsatellites, alongside previously established genetic markers, to determine the population structure of fall armyworm across Africa. Once it has been established that SfMNPV is prevalent in fall armyworm in Africa, molecular methods are combined with flight mill experiments to gain a deeper understanding of the impact of viral disease on fall armyworm flight and immunity.

Chapter One is an introduction into the many topics covered by this thesis, it focuses on insects and synthesises current knowledge on invasive species and migration, and what is known about how disease interacts with migration. It considers the role of genetics in migration, and what genetic analysis can tell us about migration in insects. It then takes an in-depth look at the fall armyworm, explaining what is currently known about it in its native and invasive regions, and highlights some of the research gaps this thesis aims to address.

The first research chapter (Chapter Two) addresses an important question by asking which microbial natural enemies are present in fall armyworm in Africa. This fills a large gap in the current knowledge of fall armyworm, with much of the research on natural enemies focusing on parasitoids and predators. This confirms that four microbial natural enemies are present in fall armyworm which are SfMNPV, SpexNPV, *M. rileyi* and *Wolbachia*. The prevalence of each disease varies significantly between countries. An in-depth look at SfMNPV reveals that this could be due to variation in temperature, rainfall, elevation, growing season, and the time since fall armyworm first invaded that country. This research reveals for the first time that SpexNPV and *Wolbachia* can be present as covert infections in

fall armyworm. These findings highlight the importance of studying the presence of microbial natural enemies in fall armyworm and understanding where they are present.

To further understand fall armyworm population structure and if it could help explain the distribution of natural enemies, Chapter Three uses larval samples collected in six African countries (Ghana, Kenya, Malawi, Rwanda, Sudan and Zambia) to determine population structure by using microsatellites. This microsatellite approach, which has not been used on invasive fall armyworm before, is compared to traditional genetic markers (*Tpi* and *COI*) used to study population structure in fall armyworm. These findings reveal slight differentiation between some countries, but that high levels of genetic mixing are occurring between all populations of fall armyworm in Africa. Knowing that high levels of population mixing is occurring between fall armyworm in different countries could have important implications in natural enemy prevalence. Viruses can be vertically transmitted and some evidence is presented that suggests that SfMNPV distribution might be influenced by which populations mix more frequently. The improved understanding of population movements provided by Chapter Three will be important in understanding fall armyworm populations and risks, such as how pesticide resistance could spread is important as this has consequences on pest control methods.

Considering the wide prevalence of SfMNPV in Africa, and its potential for biocontrol, Chapter Four aims to learn more about the effects of SfMNPV on fall armyworm flight at the molecular level by using RNA-sequencing. This chapter takes an in-depth look into gene expression in each sex, making comparisons between males and females to determine how each sex responds to the stress of infection and flight. Understanding what is going on at the molecular level can greatly enhance our knowledge of the reasons behind observed behaviours. This molecular approach revealed differences in gene expression related to reproduction, immunity, metabolism and ROS protection between male and female fall armyworm following flight when infected with SfMNPV.

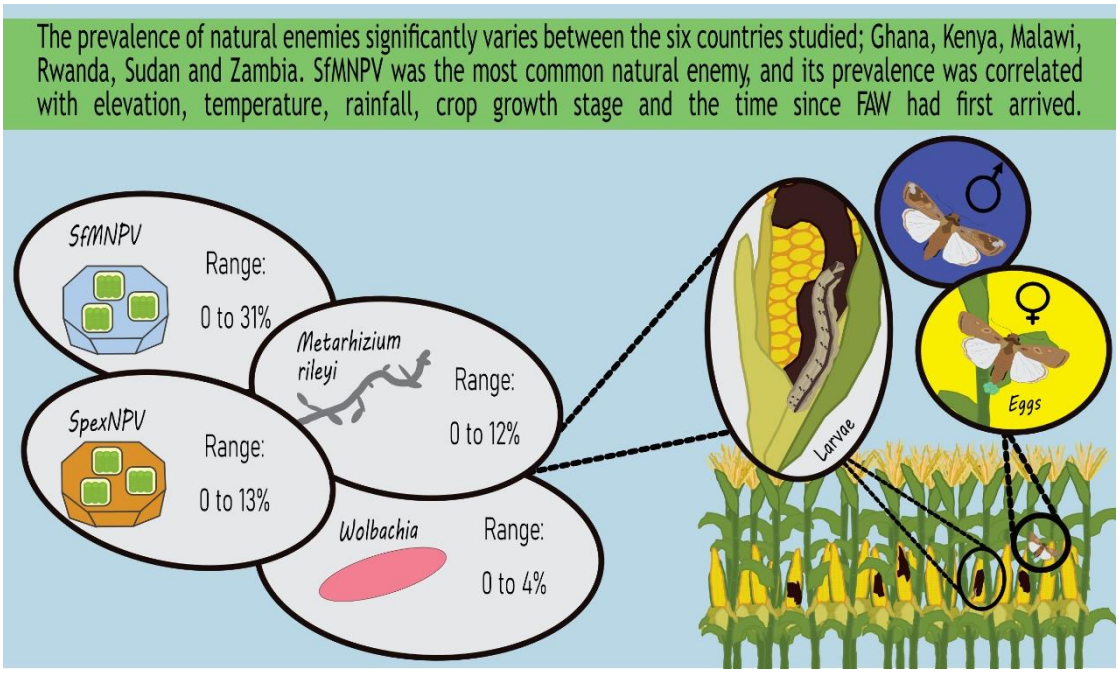
Chapter Five aims to further clarify the effects of SfMNPV on fall armyworm immunity following flight. Four genes from the immunity-related Toll-pathway were selected that were identified in the Chapter Four as being differentially expressed during flight; serine protease, MYD88, cactus and defensin. This approach allowed clarification of the expression of these genes in response to SfMNPV and flight by including more experimental groups; flown and not-flown, SfMNPV-challenged and not challenged, male and female. This revealed important differences in immune input between flown and not flown fall

armyworm, revealing the ability to control immune gene expression to favour flight. Additionally, confirmed that males and females do invest differently in immunity when exposed to the stressors of viral challenge and flight.

Finally, Chapter Six brings the four research chapters (Chapters Two to Five) together to discuss and consolidate the knowledge this thesis has contributed to the current field. This approaches the discussion from two sides; molecular and behavioural. It explains the contribution this thesis has brought to both of these fields independently, and then moves on to consolidate this information. Finally, real-world applications of this thesis are discussed that could contribute to improving control of fall armyworm in Africa, and if this can be generalised to the other continents and invasive species.

Overall, throughout this PhD the aim is to decipher the complex interactions between migration and disease. It considers how these can influence agricultural practices through understanding crop pest movements and the role natural enemies have in population control. It identifies that these interactions are different for males and females, and discusses the evidence of this. Combined, this thesis greatly improves our current understanding of fall armyworm by using a range of field, behavioural and molecular based experiments.

Chapter 2 - The distribution of covert microbial natural enemies in fall armyworm in Africa.



2.1 Abstract

Natural enemies have an important role to play in controlling pest populations, however, in invasive populations the presence and prevalence of natural enemies is generally unknown in the early stages of invasion. This study addresses this issue for the invasive fall armyworm (FAW, *Spodoptera frugiperda*) in Africa by screening larval samples from Malawi, Rwanda, Kenya, Zambia, Sudan, and Ghana for the presence of four different microbial natural enemies; *Spodoptera frugiperda Nucleopolyhedrovirus* (SfMNPV), *Spodoptera exempta NPV* (SpexNPV), the fungal pathogen *Metarhizium rileyi* and the bacterium *Wolbachia*. All four microbes were found to be present in FAW in Africa, and this study provides the first evidence of SpexNPV and *Wolbachia* infecting FAW in the field. It is likely that SfMNPV arrived with FAW from the Americas, but for the endemic SpexNPV and *M. rileyi,* this is the first evidence of host spillover from *Spodoptera exempta* to FAW. *Wolbachia* has not previously been recorded in FAW in the Americas, but it is usually transmitted vertically so it may have arrived with FAW from the Americas, alternatively, it could have infected FAW through parasitoid horizontal transmission from the African armyworm. The most prevalent pathogen was SfMNPV. In some countries, the prevalence of SfMNPV increased with the time since FAW had first been recorded in the country, although infection levels tended to decline through the growing season. The prevalence of SfMNPV was also correlated with elevation, temperature, and rainfall, with SfMNPV declining at increasing elevations and temperatures, and increasing with the frequency of rainfall. Overall, this study shows that microbial natural enemies are present in Africa and could help to control populations of newly invasive pests, although the level of control is likely to be affected by temporal and geographic factors.

2.2 Introduction

Natural enemies play an important role in the control of crop pests. They can reduce population size, which leads to improved crop yields and economic benefits (Zhang and Swinton, 2009). They have the potential to make a significant contribution to achieving the United Nations Sustainable Development Goals such as reducing food shortages, improving food security, and promoting sustainable agriculture (United Nations, 2020). An indirect benefit of natural enemies is the reduced need for biochemical inputs such as insecticide sprays which benefit the farmer economically through improved yields and reduced pesticides costs, as well as lowering carbon dioxide (CO_2) emissions, and preventing losses of non-target species (Zhang and Swinton, 2009; Heimpel et al., 2013; Jepson et al., 2014; Andersson and Isgren, 2021).

Although the role of natural enemies in controlling native pests is well documented, much less is known about their role in controlling newly invasive species, and this is especially true for naturally occurring microbial pathogens such as viruses, bacteria, and fungi. It is important to understand natural enemies in invasive species due to the enemy release hypothesis. This hypothesis posits that once in a new environment a newly invasive species is exposed to fewer natural enemies and is unlikely to be controlled by native enemies that are not yet familiar with the new host (Cornell and Hawkins, 1993; Torchin and Mitchell, 2004). However, this effect may diminish over time as natural enemies adapt to the presence of the newly invasive pest as established invasive species have up to six-times more pathogens compared to more recently introduced invasive species (Mitchell et al., 2010).

In addition to time since first incursion, there are many other factors that affect the prevalence and distribution of natural enemies and understanding these can be vital for our knowledge of host population dynamics and pest management. For example, rainfall, humidity, and temperature may influence the presence of entomopathogenic fungi and nucleopolyhedroviruses (NPV) (Fuxa and Richter, 2001; Alyokhin et al., 2011; Hajek and Tobin, 2011). This suggests that these pathogens might successfully reduce the population of an invasive species in one location but not another. Tri-trophic interactions between plants, pathogens and herbivore pests means the abundance of pathogens in crop pests can be affected by their host plant (Agrawal, 2000; Cory and Hoover, 2006). These interactions can occur through many mechanisms, such as insect immune defences changing in response to crop age, plant hormones, prior wounding of the crop, crop type and crop age (Inyang et al., 1998; Ali et al., 1998; Shikano et al., 2010; Shikano et al., 2017a; Shikano et al., 2017b).

To address this lack of evidence, this study investigates microbial natural enemies present in the fall armyworm (*Spodoptera frugiperda*, FAW), a highly invasive crop pest. Native to America, FAW spread to Africa in 2016, and by 2018 it was present in at least 44 African countries. From late 2018 and into 2019 it continued its rapid spread into Asia, and then Australasia in 2020. The FAW is a generalist species, meaning it can feed on a wide variety of plants. However, it predominately targets rice and maize, which are staple foods in much of Africa and Asia (Devi, 2018, Chhetri and Acharya, 2019, Pashley, 1986). The impact of these new incursions can lead to huge crop losses, for instance, in 2018 83% of Kenyan maize farmers were affected by FAW and around 1 million tonnes of maize were lost (De Groote et al., 2020). This in turn has an economic impact and can result in severe food shortages, especially in countries where many farmers are smallholders that rely on their crops for subsistence. Improving our knowledge of how microbial natural enemies' control FAW population dynamics can help to mitigate against this damage, and improve our understanding of the role microbial natural enemies may have in controlling invasive pest species more generally.

In the native range of FAW, many different natural enemies have been documented. In Mexico, 30% of larvae collected from maize were targeted by a natural enemy, including seven parasitoids, two entomopathogenic fungi and the baculovirus *Spodoptera frugiperda nucleopolyhedrovirus* (SfMNPV) (Virgen et al., 2013). By contrast, very little is currently known about which natural enemies are presently attacking the pest in its introduced range. In some African countries, such as Ghana, Benin, Ethiopia, Tanzania, Uganda, and Kenya, parasitoids have been found that lay eggs in FAW eggs or larvae which then emerge and kill FAW; this can help to control populations (Sisay et al., 2018; Sisay et al., 2019; Agboyi et al., 2020; Otim et al., 2021). The number of parasitoids identified ranges from 3 to 13 across countries, and includes species such as *Chelonus bifoveolatus, Cotesia icipe* and *Coccygidium luteum* (Sisay et al., 2018; Sisay et al., 2019; Agboyi et al., 2020; Otim et al., 2021). However, very little research has been carried out into which entomopathogens are present in Africa. The present study aimed to expand this knowledge gap by screening FAW larvae for the presence of microbial natural enemies, specifically SfMNPV and *Spodoptera exempta nucleopolyhedrovirus* (SpexNPV), the fungus *Metarhizium rileyi*, and the bacterium *Wolbachia*.

In the native range of FAW, SfMNPV is the most common natural enemy, present in 11% of larvae sampled in Nayarit, Mexico (Virgen et al., 2013). Overt SfMNPV disease was also present in invasive FAW collected in the Hubei province of China (Lei et al., 2020), and

an isolate was extracted from FAW collected in the Sudan-Guinea savannah in Nigeria (Wennmann et al., 2021). However, SfMNPV has not yet been confirmed in FAW in East and Central Africa, and its prevalence in Africa is undetermined. In addition to being natural enemies, NPV are used for biocontrol so understanding natural levels within a population can help farmers within integrated pest management programs. The advantage of using SfMNPV to control crop pests is that they have no effects on non-target species as they specialise in infecting lepidopteran hosts, whereas spraying maize with common insecticides can reduce the abundance of natural enemies and non-target species (Armenta et al., 2003; Gómez et al., 2013). SfMNPV is widely used in the Americas to control FAW and it is currently in the process of being registered for use in seven sub-Saharan African countries following successful field trials (Bateman et al., 2021). It is hoped that increasing the understanding of natural SfMNPV levels within Africa will help with registering and licensing of biocontrol agents to help mitigate against FAW.

NPVs are typically highly species-specific, however, previous work has shown that the specific NPVs of *Spodoptera exigua*, *Spodoptera littoralis* and FAW are able to initiate infection in all three host species (Simon et al., 2004). In Africa, SpexNPV is present naturally in populations of *Spodoptera exempta,* also known as the African armyworm. The prevalence of SpexNPV in *S. exempta* in Tanzania was 60% in larvae that had transcriptionally active virus, with 78-100% being PCR positive for SpexNPV (Vilaplana et al., 2010). SpexNPV is being developed for use as a biocontrol agent to help control outbreaks of *S. exempta* (Grzywacz et al., 2008). There is currently no evidence that SpexNPV can infect any other species apart from its specific target, however, considering its high abundance in Africa and the potential for species spillover, in this chapter FAW larvae were screened for the presence of SpexNPV to see if it can infect FAW in the wild.

Wolbachia is a gram-negative bacterium that is an obligate symbiont of many insects that can be parasitic or beneficial (Floate et al., 2006). *Wolbachia* is present within *S. exempta* populations in Africa, with up to 56% of larvae infected and it could be driving haplotype diversity and changing sex ratios as a male-killer strain has been identified (Graham & Wilson 2012; Graham *et al.* 2012). The presence of *Wolbachia* within *S. exempta* can increase larval susceptibility to SpexNPV, leading to higher mortality rates and lower lethal dose thresholds (Graham et al., 2012). *Wolbachia* is present in the native range of FAW, found in about 1% of Lepidoptera and Papilionoidea species in South America and 31% in North America (Ahmed et al., 2015a). However, *Wolbachia* has not previously been detected in FAW in the Americas (Dumas et al., 2015). It is important to establish whether it

is present in African FAW as it can interact with biocontrol agents (Graham et al., 2012), and affect population dynamics through mechanisms such as male-killing and reducing fecundity (Floate et al., 2006).

Metarhizium rileyi is a pathogenic fungus that predominately infects Lepidoptera, with around 60 known vulnerable species (Fronza et al., 2017). *M. rileyi* occurs in the USA and South America, however, documented cases of it infecting FAW in the field in its native region are rare (Ignoffo and Garcia, 1985; Fronza et al., 2017; Álvarez et al., 2018). High pathogenicity and species-specificity make *M. rileyi* an ideal candidate for biological control, especially in countries that have suitable climates (high relative humidity, temperatures of around 25°C and rainfall) such as Brazil, India and Sub-Saharan Africa (Fronza et al., 2017). *M. rileyi* is present across Africa and infects the closely related *S. exempta* (Rose et al., 2000). *M. rileyi* has been found as a natural enemy in invasive FAW populations in China (Zhou et al., 2020) and India (Mallapur et al., 2018; Sharanabasappa et al., 2019; Firake and Behere, 2020). The presence of *M. rileyi* can be particularly beneficial for biocontrol, with *M. rileyi* and SfMNPV responsible for around 50% of mortalities in North East India (Firake and Behere, 2020). *M. rileyi* has not been officially recorded in Africa on FAW, though fungal infections have been reported in field populations of FAW in Malawi and Zambia (Food and Agriculture Organisation, 2018). This chapter increases the current understanding of *M. rileyi* in Africa by screening samples for covert infections, as well as sequencing DNA from larvae with an overt fungal infection to confirm the species.

To further improve current understanding of natural enemies of the fall armyworm, this study aimed to establish whether four important entomopathogens are present in FAW in Africa; SfMNPV, SpexNPV, *M. rileyi* and *Wolbachia*. Following the discovery of SpexNPV in FAW, a bioassay was carried out to determine the impact of SpexNPV infection. Finally, we determine how the prevalence of SfMNPV is affected by a range of environmental features previously shown to influence pathogen levels, including weather, elevation, time since the FAW invasion and the crop development stage.

2.2.1 Hypotheses

This chapter tests the following hypotheses by screening field samples of FAW larvae for microbial natural enemies and modelling the environmental variables that may affect prevalence:

1. SfMNPV will be present in natural populations of FAW as it is present in high numbers in the source populations in the Americas, and in invasive populations in China and Nigeria (Wennmann et al., 2021; Lei et al., 2020).
2. *M. rileyi* will be present in natural populations of FAW as it is present in the source populations in the Americas, and in invasive populations in China and India, as well as in being present in high numbers in the closely related *S. exempta* in Africa (Rose et al., 2000; Fronza et al., 2017; Mallapur et al., 2018; Zhou et al., 2020).
3. SpexNPV will not be present in natural populations of FAW due to its high host-specificity.
4. *Wolbachia* will not be present in natural populations of FAW as it has not previously been detected in FAW in source populations in the Americas and is usually passed on via vertical transmission.
5. The presence of SfMNPV, *M. rileyi,* SpexNPV and *Wolbachia* will vary between locations within countries, and between countries, due to changes in environmental conditions, agroecological zones and the time since FAW first arrived.
6. The prevalence of SfMNPV will increase with the length of time since the first record of FAW in the country.
7. Time into the growing season (i.e., the time since the crop was sown) will influence SfMNPV prevalence as it affects the plant crop as well as population dynamics of FAW. It is hypothesised that prevalence will increase later in the growing season as FAW may be more abundant and will have been through more generations (Graham and Wilson, 2012; Chapman et al., 2015).
8. Higher rainfall and temperature will increase the prevalence of SfMNPV due to the increases in FAW growth and the spread of virus particles (Fuxa and Richter, 2001; Alyokhin et al., 2011; Hajek and Tobin, 2011).

2.3 Methods

2.3.1 Sample collections

FAW larvae were collected from various locations in Malawi, Rwanda, Kenya, Zambia, Ghana, and Sudan (Fig 2.1, Table 2.1). Following collection larvae were stored in individual tubes with ethanol for transport to the UK. On arrival, the tubes with larvae in were stored at -20°C.

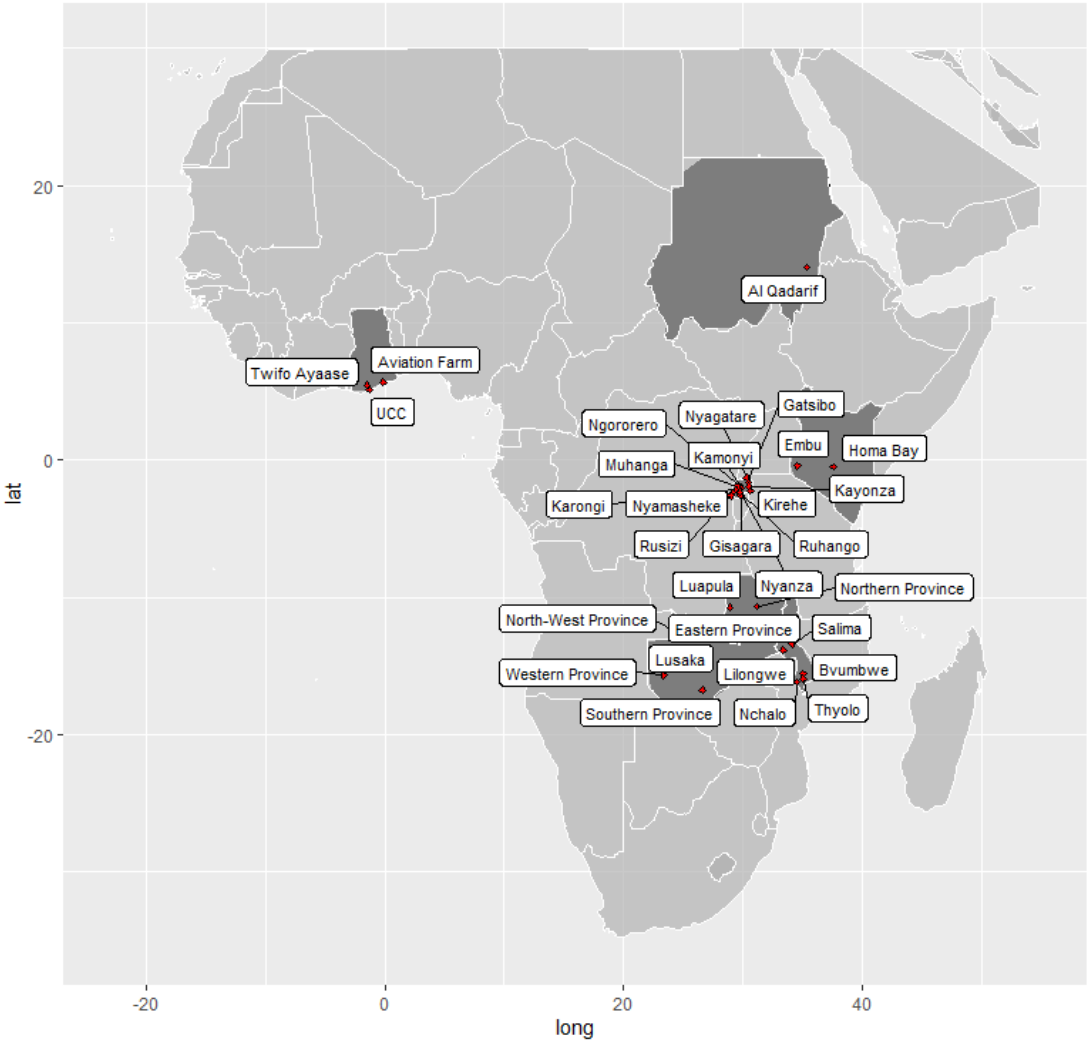


Figure 2.1 Sampling locations of *S. frugiperda* larvae. Larvae at third or fourth instar were collected in various locations between 2017 and 2019. The collection locations are shown on the map and were in Ghana (N=72), Kenya (N=39), Malawi (N=181), Rwanda (N=125), Sudan (N=28), and Zambia (N=79).

Table 2.1 Fall armyworm larvae collection details. All larvae were collected at the third or fourth instar. If X (longitude) and Y (latitude) were not recorded, then it was estimated using a central point for the region as this information was needed for geographic and temporal analysis of SfMNPV.

Country	Location	Collector*	N	Crop	Date	X	Y
Ghana	Aviation Farm	A	4	Maize	16/10/2017	-0.16	5.68
Ghana	Twifo Ayaase	A	30	Maize	14/08/2017	-1.49	5.47
Ghana	UCC	A	38	Maize	22/12/2017	-1.29	5.11
Kenya	Embu	B	9	Maize	19/06/2019	34.58	-0.43
Kenya	Homa Bay	B	30	Maize	19/06/2019	37.60	-0.49
Malawi	Bvumbwe	C	40	Maize	23/01/2019	35.04	-15.55
Malawi	Lilongwe	C	31	Maize	28/01/2019	33.38	-13.85
Malawi	Nchalo	C	40	Maize	17/09/2018	34.55	-16.16
Malawi	Salima	C	30	Maize	28/01/2019	34.15	-13.40
Malawi	Thyolo	C	40	Maize	17/09/2018	35.07	-15.92
Rwanda	Gatsibo	D	10	Maize	04/05/2017	30.47	-1.58
Rwanda	Gisagara	D	8	Maize	05/05/2017	29.85	-2.59
Rwanda	Kamonyi	D	10	Maize	05/05/2017	29.90	-2.01
Rwanda	Karongi	D	10	Maize	04/05/2017	29.42	-2.16
Rwanda	Kayonza	D	10	Maize	03/05/2017	30.51	-1.91
Rwanda	Kirehe	D	10	Maize	03/05/2017	30.64	-2.26
Rwanda	Muhanga	D	8	Maize	05/05/2017	29.73	-1.96
Rwanda	Ngororero	D	10	Maize	05/05/2017	29.62	-1.86
Rwanda	Nyamasheke	D	10	Maize	03/05/2017	29.17	-2.38
Rwanda	Nyanza	D	9	Maize	05/05/2017	29.75	-2.35
Rwanda	Nyagatare	D	10	Maize	05/05/2017	30.33	-1.29
Rwanda	Ruhango	D	10	Maize	10/05/2017	29.78	-2.31
Rwanda	Rusizi	D	10	Maize	03/05/2017	29.01	-2.58
Sudan	Al Qadarif	F	28	Sorghum	01/09/2017	35.38	14.04
Zambia	Eastern Province	E	4	Maize	18/01/2017	32.42	-12.91
Zambia	Luapula	E	9	Maize	15/05/2017	28.93	-10.71
Zambia	Lusaka	E	15	Maize	20/01/2017	28.32	-15.40
Zambia	Northern Province	E	10	Maize	15/05/2017	31.19	-10.65
Zambia	North-West Province	E	7	Maize	15/05/2017	25.16	-12.86
Zambia	Southern Province	E	3	Maize	21/01/2017	26.62	-16.73
Zambia	Western Province	E	3	Maize	20/01/2017	23.38	-15.68

*A: Ben Mensah, B: Aislinn Pearson, Sevgan Subramanian and Kentosse Gutu Ouma, C: Donald Kachigamba and Amy Withers, D: Patrick Karangwa and Bellancile Uzayisenga, E: Gilson Chipabika and Miyanda Moonga, F: Guillaume Sneessens

2.3.2 DNA extraction

DNA was extracted from the tail half of all larvae (≤25mg) following the standard protocol for tissue in the Qiagen DNeasy Blood and Tissue kit (Qiagen 2018). DNA was stored in 100µl buffer AE (10 mM Tris-Cl 0.5 mM EDTA; pH 9) at -20°C. The protocol was altered slightly for extracting DNA from larvae collected in Sudan, these modifications were 200µl ATL and an additional 200µl 1x SSC before incubation and the DNeasy Spin Column was centrifuged at 13,000 RPM.

2.3.3 Covert infection detection

Samples were screened for the presence of covert SpexNPV, SfMNPV, *M. rileyi* and *Wolbachia* using standard PCR. Samples were originally collected for strain identification and stored in ethanol, therefore they were unsuitable for RNA extraction so only presence or absence data of viral DNA could be collected. The primer sequences and cycling parameters for each infection tested are shown in Table 2.2. Each PCR reaction was carried out in a 0.2ml PCR tube and the reagents were 1µl EasyTaq® Buffer (Transgen biotech), 0.5µl 10µM forward primer, 0.5µl 10µM reverse primer, 0.2µl dNTPs (Transgen biotech), 0.05µl EasyTaq® DNA Polymerase (Transgen biotech), 5.75µl H_2O and 1µl DNA. Each PCR was carried out twice as the pathogen DNA may be at very low concentrations, except for *M. rileyi* which was only carried out once. To visualise DNA, electrophoresis was used with samples loaded into a 1% agarose gel at 125 volts for 45 minutes. If a band was visible in either PCR repeat then infection in the sample was recorded as present; if no band was visible then the sample was recorded as not infected. Species specificity of the SfMNPV and SpexNPV primers was confirmed using DNA extracted from larvae with known overt infections (Appendix B, Table S2 and Fig. S1).

Table 2.2 Primer information for each microbial natural enemy. These primers were used for the detection of microbial natural enemies in this study.

Primer	Expected product size (bp)	F primer sequence	R primer sequence	Cycling parameters
Spodoptera exempta nucleopolyhedrovirus: orf 57-58 (Donkersley P., unpublished)	300	5'-GTCGTGCAGTTCCTTGTAGT	5'-ACAAGACAAACGACAATGTGTG	ID: 95ºC 2min D: 95ºC 30sec } A: 60ºC 30sec } 30 cycles E: 68ºC 45sec } FE: 68ºC 5min
Spodoptera frugiperda nucleopolyhedrovirus: Sfpg41.1 gene (Simón *et al.* 2008)	650 – 750 (some variation between genotypes)	5'-CGACAATGTCATCGTCTTCG	5'-ATATGTTAGTGGTGGCGGAC	ID: 95ºC 2min D: 95ºC 30sec } A: 52ºC 30sec } 30 cycles E: 68ºC 45sec } FE: 68ºC 5min
Wolbachia (Zhou, Rousset & O'Neil 1998)	590 – 632 (some variation between genotypes)	5'-TGGTCCAATAAGTGATGAAGAAAC (Wsp81F)	5'-AAAAATTAAACGCTACTCCA (Wsp691R)	ID: 94ºC 5min D: 94ºC 30sec } A: 52ºC 30sec } 40 cycles E: 72ºC 45sec } FE: 72ºC 5min
Metarhizium rileyi (Tseng *et al.* 2010)	284	5'-CCAAGCCACCAGTCAATTTC (NS1)	5'-TATCACCAGCCTCGATCACC (NS2)	ID: 95ºC 2min D: 95ºC 30sec } A: 56ºC 30sec } 30 cycles E: 68ºC 45sec } FE: 68ºC 5min
Universal fungal primers EF1-1002 (Stielow *et al.* 2015)	1000	5'-TTCATCAAGAACATGAT	5'-GCTATCATCACAATGGACGTTCTTGAAG	ID: 94ºC 10min D: 94ºC 1min } A: 52ºC 1min } 33 cycles E: 72ºC 1min } FE: 72ºC 10min

2.3.4 Identification of overt fungal infections in FAW

Larvae samples with signs of fungal infection were collected on maize crops in Zambia. Seven larvae samples had DNA extracted using the standard protocol for the Qiagen DNeasy Blood and Tissue kit (Qiagen, 2018). DNA was amplified using universal fungal primers for EFI, the primer sequences and cycling parameters are shown in Table 2.2. Amplification was carried out in 20 µl reactions with 1 x platinum master mix with an additional 1mM MgCl2, 0.5µM of each primer and 100-300 ng template DNA. Amplicons were purified with Microclean and sequenced using the BigDye terminator v3.1 sequencing kit.

2.3.5 Bioassay of SpexNPV in FAW

Following the detection of SpexNPV in larvae in Africa, a bioassay was carried out to determine potential effects of the virus in FAW. As it was not possible to isolate the specific SpexNPV detected in larvae for this study, a stock solution of SpexNPV isolated from African armyworm was used instead to give an indication of potential effects of the virus on FAW. A lab culture of FAW was used in this bioassay collected from the field in Zambia three months prior to the bioassay. For this bioassay, 210 FAW neonates were starved for 2 hours in 96-well plates, larvae were fed a 1µl droplet of diluted SpexNPV at a concentration of 0 to 10^7 OBs/ml (occlusion bodies per ml). For each concentration 35 larvae were used. The viral dose was achieved by diluting with blue food colour, dH_2O and 30% sugar water to ensure the diet was still palatable for the larvae. They were left for 15 minutes and then neonates with a visibly blue gut were placed into individual diet pots with an excess of artificial diet (diet details in Appendix B, Table S3). Larvae that died within 24 hours were recorded as 'handling' deaths and were removed from the experiment. The final number of larvae per experimental group are in Table 2.6. Larvae were monitored daily for mortality and development. Growth was recorded as stunted for individuals that were two or more instars smaller than the rest of the larvae throughout the experiment. Diet pots were changed as necessary. SpexNPV was provided by David Grzywacz at the National Resources Institute at the University of Greenwich.

2.3.6 Geographical, meteorological, and temporal data selection

The variables included in the model are briefly described in Table 2.3, with a full description and information on how they were obtained below. The mean and standard deviation of each variable for each country is shown in Table 2.4.

2.3.6.1 Rainfall, temperature and SfMNPV: development days prior to collection

Rainfall and temperature data were obtained from Copernicus Climate Change Service and was based on hourly ECMWF ERA5 data at surface level for the development days prior to FAW larvae collection (Boogaard and Grijn, 2021). Average lifecycle of FAW from egg to adult varies with temperature (Du Plessis et al., 2020). Monthly mean temperatures were obtained for the month the sample was collected in. This was then used to estimate development time of FAW around that time of year (Du Plessis et al., 2020), rounding to the nearest data point available or using the median if the mean temperature was in the middle of two data points. The number of development days was then used to obtain the weather data for the same number of days, for example, at 26°C the mean time of development from egg to larvae was 29 days, so the data included in the model was for the 29 days prior to sample collection.

2.3.6.2 Elevation

Elevation data was extracted for each sample point using the World_Topo_Map from the ArcGIS Map Service (ArcGIS, 2021) in ArcGIS Pro.

2.3.6.3 Time

The dates of FAW recorded in each country can vary, based on official reports and when the recording was confirmed. Therefore, we ran the model for SfMNPV prevalence for all countries using two measures of time. These were the number of days since FAW had first been recorded in Africa (*days since Africa*) and the number of days since FAW had first been recorded in each individual country (*days since country*). There was no significant difference between the models (χ^2_{29}=89.03, *P*=0.504) and *days since country* had a lower AIC (122.97) than *days since Africa* (123.48). Additionally, days since FAW was first recorded in the country showed more variation: Zambia – November 2016, Malawi - December 2016, Kenya and Ghana - March 2017, Rwanda - May 2017 and Sudan – September 2017 (Uzayisenga et al., 2018; Wilson, 2021). Considering this, days since country was selected as the variable to include in the statistical analysis.

2.3.6.4 Growing season

Crop growing season for each country was based on the FAO Crop Calendar for Malawi, Ghana, Kenya, Zambia and Sudan, and on the 2017 Seasonal Agricultural Survey for Rwanda (FAO, 2010; Rwanda, 2017). Rainfall accumulation was calculated in 10-day blocks from the start date of the crop growing season for each sampling location (Maize for Malawi, Rwanda, Ghana, Kenya and Zambia, Sorghum for Sudan). For each sampling location, the start of the

growing season was designated as the date that >25mm of rain had accumulated in the previous 10 days. This is because rainfall is linked to crop growth, and >25mm in a 10 day period typically triggers planting within the growing season for maize (Tadross et al., 2005; Tadross et al., 2009). If this method gave the days since the start of the growing season as >5 days longer than crop cycle period, then the latest date of the crop sowing period was used instead.

Table 2.3 The variables used in the models of factors affecting SfMNPV prevalence in FAW. The geographical, meteorological, and temporal variables used in this study to determine their effects on SfMNPV prevalence in FAW, with the name they were given for the analysis and a brief description of each variable. The final selected variables for use in analyses were *Days_with_rain, Mean_temp, Elevation_m, Days_since_country,* and *Days_since_growing_season.*

Variable	Description
Days_with_rain	The number of days with >1mm rainfall during the development time of FAW.
Mean_temp	The mean temperature of the development time of FAW.
Elevation_m	The elevation (m) of the sampling location.
Days_since_Country	The number of days before the sampling date since FAW had first been recorded in each country.
Days_since_Africa	The number of days before the sampling date since FAW had first been recorded in Africa.
Days_since_growing_season	The number of days into the crop growing season it was when sampling occurred.

Table 2.4 The mean and standard deviation (sd) for each variable for each country. Sudan only had one sampling location so the mean and standard deviation could not be calculated.

Variable	Measurement	Ghana	Kenya	Malawi	Rwanda	Sudan	Zambia
Temperature (°C)	mean	25.24	21.2	22.74	18.73	27.78	21.98
	sd	1.17	1.28	2.89	1.45	NA	1.38
Days with rain	mean	24.33	36	24.2	46.69	10	28.86
	sd	5.77	0	20.36	12.08	NA	15.74
Elevation (m)	mean	71	1248.5	768.4	1633.15	599	1153.14
	sd	66.2	47.38	394.4	220.95	NA	163.41
Days since growing season	mean	104.67	68.5	80.6	51.23	53	103.14
	sd	20.03	17.68	26.24	5.97	NA	60.05
Days since country	mean	230.33	840	733.8	33.62	1	111.00
	sd	65.01	0	71.96	1.85	NA	59.37

2.3.7 Statistical analysis

2.3.7.1 Identifying differences in the prevalence of SfMNPV, Wolbachia, *M. rileyi* and SpexNPV

Each entomopathogen and country was analysed individually to determine if there was a significant difference in the presence of each infection between countries and between the sampling locations within countries. Including all countries and sampling locations in a single model meant the model was unstable due to limited sample size and the absence of pathogens in some locations and countries, therefore each country was analysed individually to detect within-country variation between sampling locations. Statistics were carried out in R (v. 4.0.3) (R Core Team, 2020). A weighted binomial glm was carried out (glm = (family=binomial) function) on the presence and absence values with locations within and between countries, and a likelihood ratio test (anova(test= "Chi^2") function) was used to obtain the reported *p* values for each variable. Using the presence and absence values in the model meant the model was weighted based on the number of larvae collected as this varied between sampling locations (Table 2.1).

2.3.7.2 The effects of SpexNPV in FAW

The effect of SpexNPV dose on stunted growth was tested using a linear model with family as quasibinomial in R (v 4. 0. 3).

2.3.7.3 Principal components analysis (PCA) for the effects of environmental variables on SfMNPV prevalence

There was strong correlation between the variables (mean temperature, days with rain, elevation, days since start of growing season and days since FAW was first recorded) in this study (Fig. 2.2, Appendix B, Fig. S2 shows correlation of variables in Malawi alone). This was expected as crop growing season is inextricably associated with rainfall and temperature, that in turn depends on geographical features such as elevation. Due to the strong correlation between multiple variables, a PCA was used to look at the differences between the countries where samples were collected, and how SfMNPV distribution might be linked to these variables. A PCA was carried out in R (v4.0.3) on the five variables described above (days with rain, mean temperature during development period, elevation of sampling location, days since FAW was first recorded in the country, days into growing season when sampling occurred). Principal components (PC) were then extracted for a binomial glm (glm = (family=binomial) function) to look at SfMNPV prevalence in Africa.

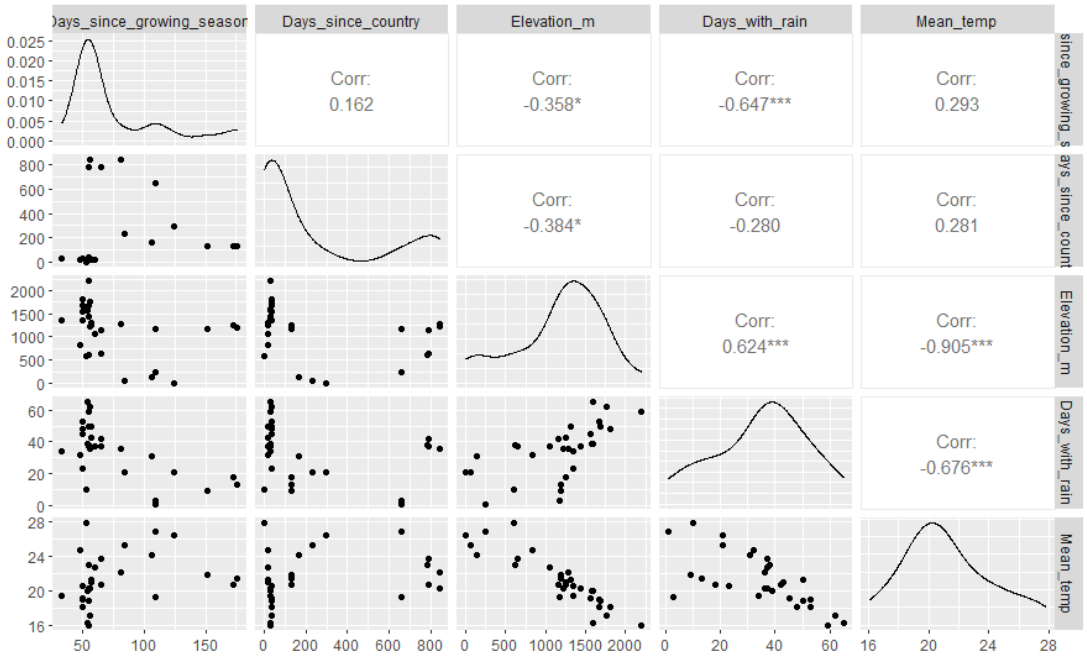


Figure 2.2 A comparison of the variables with correlation coefficients. This showed significant correlation between variables, particularly elevation and weather which means considering variables cannot be considered individually. Significance is shown with asterisk where *** indicates *P<0.001,* ** indicates *P<0.01* and * indicates *P<.0.05*.

2.3.7.4 Africa modelling for SfMNPV prevalence

A weighted binomial glm was carried out for SfMNPV prevalence for the five variables without including country in the model. This was done to detect variation due to environmental variables and exclude differences due to country-level prevalence. Seventeen models were compared using the *MuMin* package in R based on Akaike Information Criterion corrected for small sample size (AICc) (Bartoń, 2013). AICc provides an estimate of how much information is included in the model by estimating how much information is lost, therefore the lower the AICc the better the model fits the data. Pseudo R^2 was calculated using r.squaredLR function in *MuMin* package, based on the Nagelkerke method (Nagelkerke, 1991).

2.3.7.5 Malawi modelling for SfMNPV prevalence

A weighted binomial glm was carried out for SfMNPV prevalence in Malawi independently of the other countries. Fifteen models were compared using the MuMin package in R based on Akaike information criterion (AICc) corrected for small sample size, and pseudo R^2 was calculated using r.squaredLR function in *MuMin* package, based on the Nagelkerke method (Nagelkerke, 1991; Bartoń, 2013).

2. 4 Results

2.4.1 M. rileyi is confirmed to be present in FAW in Zambia

Overt fungal infection was observed in fourteen FAW larvae whilst collecting samples in Zambia (Fig. 2.3). Seven of these samples had DNA sequenced and amplified using EF1 primers to determine what fungal species was present. One sample failed to amplify a clear sequence and three samples produced mixed sequences. Of the identifiable sequences, all three were a strong match to *M. rileyi* confirming that it is present in FAW in Africa (Table 2.5, sequences in Appendix B, Table S4).

Figure 2.3 Images of covert fungal infection in FAW in Zambia. These were confirmed by sequencing EF1 to be *M.* rileyi, A) All 14 larvae collected with overt signs of fungal infection, B) image of infected larvae on maize crops. Images were taken in 2017 and provided by Ken Wilson.

Table 2.5 The top three aligned sequences for the successfully amplified EF1 sequences. Sequences were inputted into BLAST to identify the closest match, which was a 100% match to *Metarhizium rileyi* for all three sequences thus confirming that the overt fungal infections in Zambia were caused by *M. rileyi*. Images provided by Ken Wilson.

Sample		BLAST output for universal fungal sequence (EF1)				
	Scientific Name	Length of query sequence	Query Cover	E value	Percent identity	Matching accession number
	*Metarhizium rileyi*	545	99%	0	100	MH986285.1
	Metarhizium rileyi		99%	0	100	KP324764.1
	Metarhizium rileyi		99%	0	100	HQ165688.1
	*Metarhizium rileyi*	541	100%	0	100	MH986285.1
	Metarhizium rileyi		100%	0	100	KP324764.1
	Metarhizium rileyi		100%	0	100	HQ165688.1
	*Metarhizium rileyi*	563	100%	0	100	MH986285.1
	Metarhizium rileyi		100%	0	100	KP324764.1
	Metarhizium rileyi		100%	0	100	HQ165688.1

2.4.2 Does disease vary between locations within countries?

2.4.2.1 Ghana

None of the four locations sampled had SpexNPV, *Wolbachia* or *M. rileyi* present (Fig. 2.4, Table 2.6). SfMNPV was present in all four locations at varying levels of prevalence. The prevalence of SfMNPV did significantly vary between the three locations studied ($\chi^2_2 = 12.77$, P=0.002), with a 100% prevalence in the samples collected at Aviation Farm (N = 4) and just 17% of samples collected at Twifo Ayaase (N = 30).

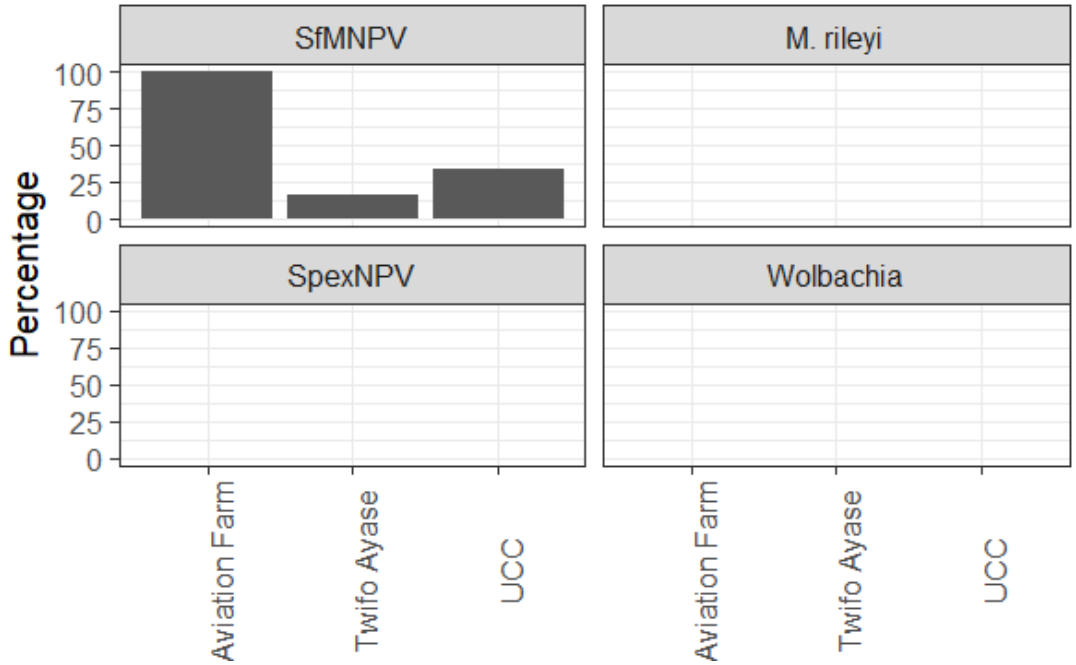


Figure 2.4 Proportion of larval samples from each location sampled in Ghana that tested positive for each microbe. No samples from Ghana were found to have *M. rileyi,* SpexNPV or *Wolbachia,* but SfMNPV was present at varying prevalences throughout the country. Sample sizes are in Table 2.5.

2.4.2.2 Kenya

SfMNPV, SpexNPV and *M. rileyi* were not present in any samples from either Homa Bay (N=9) or Embu (N = 30) in Kenya (Fig. 2.5, Table 2.6). *Wolbachia* was found in only one sample in Homa Bay and was not found in any samples from Embu. The prevalence of *Wolbachia* did not vary significantly between the two locations that were sampled in Kenya ($\chi^2_1 = 0.53$, P = 0.47).

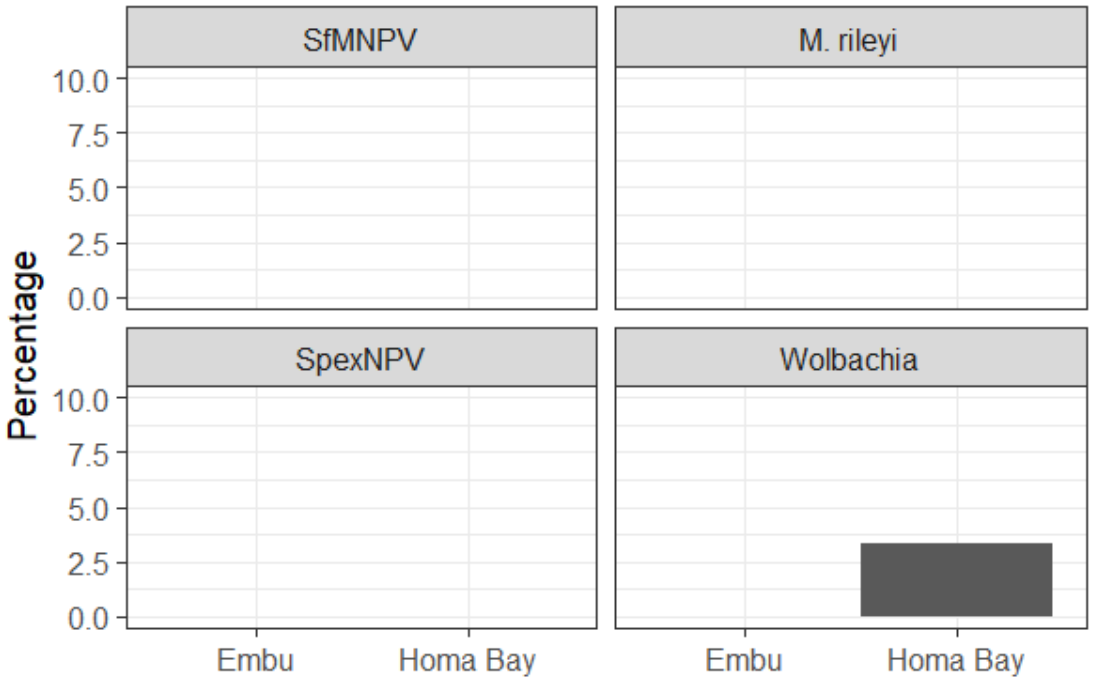


Figure 2.5 Percentage of larval samples from each location sampled in Kenya that tested positive for each microbe. The only natural enemy found in samples from Kenya was *Wolbachia,* which was only found in one location (Homa Bay). Sample sizes are in Table 2.5.

2.4.2.3 Malawi

Covert infections of all four entomopathogens were identified throughout Malawi (Fig. 2.6, Table 2.6). *Wolbachia* was the rarest infection, found only in one sample in one region (Lilongwe, N = 31) and there was no significant difference in the distribution of *Wolbachia* throughout Malawi ($\chi^2_4 = 3.56$, P=0.469). *M. rileyi* was rare, identified in only 5% of samples from Nchalo (N = 40) and 3% from Bvumbwe (N = 40). There was no significant difference in the distribution of *M. rileyi* throughout Malawi ($\chi^2_4 = 5.32$, P=0.256). SfMNPV had the highest prevalence and was present in all regions sampled. The proportion of samples positive with SfMNPV varied significantly between locations, ranging from 3% in Nchalo to 32% in Lilongwe ($\chi^2_4 = 14.04$, *P*<0.01). SpexNPV is usually only associated with *S. exempta,* however, this study found it was present in *S. frugiperda.* Its presence varied significantly between locations, and prevalence ranged from 13% in Bvumbwe, 23% in Lilongwe and 30% in Nchalo ($\chi^2_4 = 29.52$, *P*<0.01). Coinfection of both SfMNPV and SpexNPV was found in 6% of samples and varied between locations from 0% in Salima (N = 30) and Thyolo (N = 40), to 35% in Lilongwe, 30% in Bvumbwe and 8% in Nchalo.

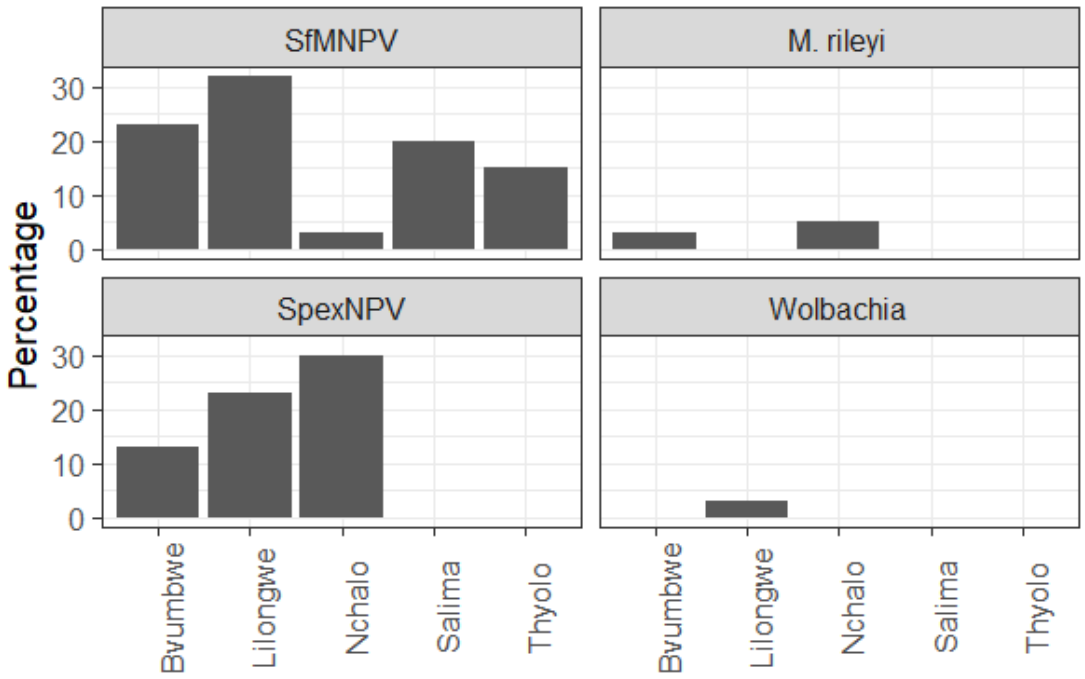


Figure 2.6: Percentage of larval samples from each location sampled in Malawi that tested positive for each microbe. All four microbial natural enemies screened for in this study were present in Malawi at varying prevalence, and each location screened had at least one of the natural enemies present. Sample sizes are in Table 2.5.

2.4.2.4 Rwanda

Most locations in Rwanda had no covert infections present, but all four natural enemies were present within the country (Fig. 2.7, Table 2.6). SfMNPV was present in samples collected in Kayonza (N = 10) and Nyagatare (N = 10), however, its prevalence did not vary significantly between locations (χ^2_{12} = 7.57, *P* =0.817). Rusizi (N = 10) was the only location where SpexNPV was present. The prevalence of SpexNPV did not vary between locations (χ^2_{12} = 16.19, *P* = 0.183). *M. rileyi* was detected in 20% of samples from Gatsibo (N = 10), however, its presence did not vary significantly between locations (χ^2_{12} = 10.56, *P* =0.56). *Wolbachia* distribution was not significantly correlated with location, and it was only identified at one location (χ^2_{12} = 5.18, *P* =0.951).

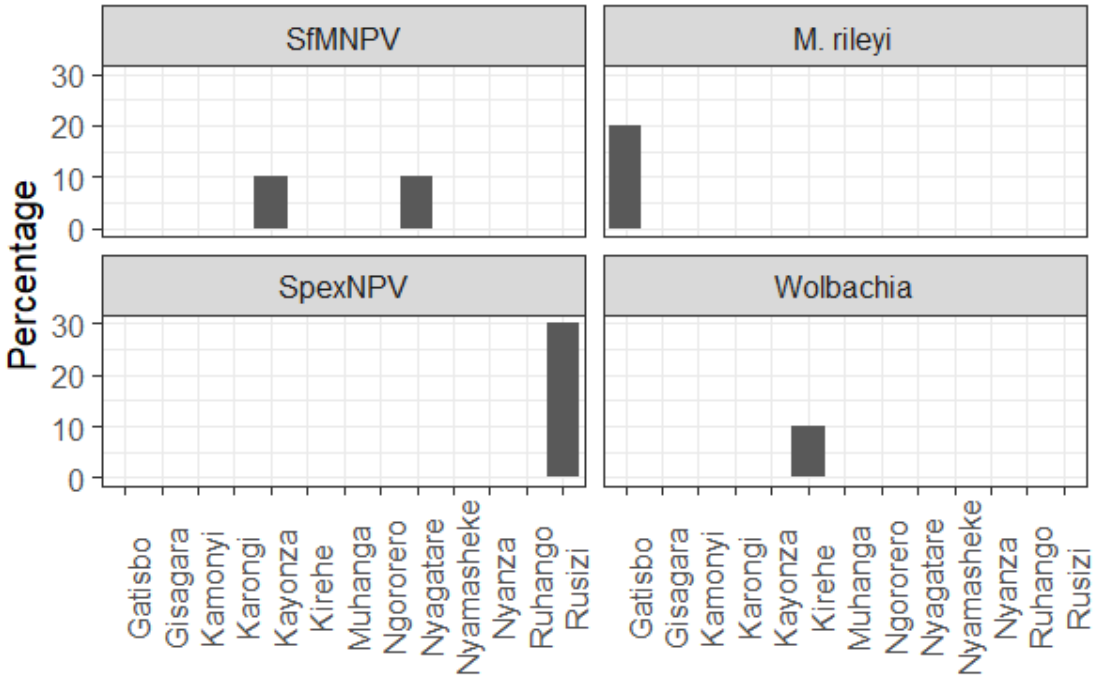


Figure 2.7 Percentage of larval samples from each location sampled in Rwanda that tested positive for each microbe. All four natural enemies were present in Rwanda at varying prevalences, and in some locations none of the four natural enemies screened for were detected. Sample sizes are in Table 2.5.

2.4.2.5 Sudan

M. rileyi and SpexNPV were not present in any larvae from Al Qadarif, Sudan (N = 28) (Fig. 2.8, Table 2.6). *Wolbachia* was present in 4% of FAW larvae. SfMNPV was the most prevalent microbial natural enemy and was present in 11% of FAW larvae.

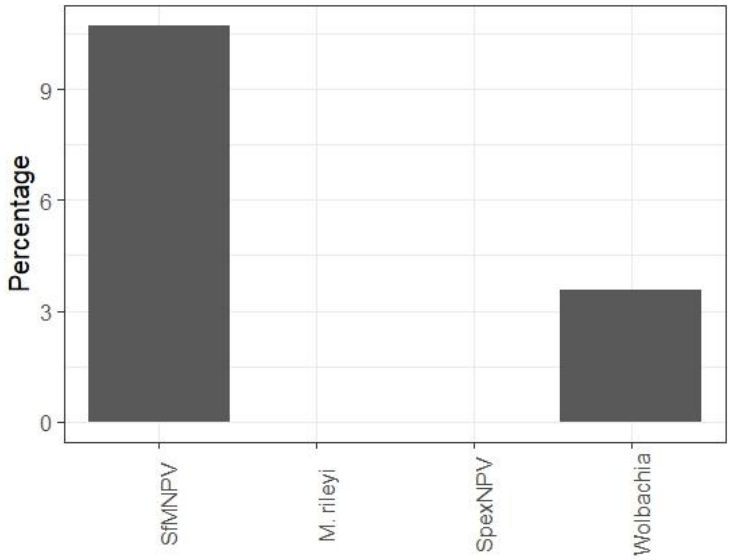


Figure 2.8 Percentage of larval samples in Al Qadarif in Sudan that tested positive for each microbe. SfMNPV and *Wolbachia* were present in Sudan, but *M. rileyi* or SpexNPV were not detected in any larvae. Sample sizes are in Table 2.5.

2.4.2.6 Zambia

SfMNPV, SpexNPV and *Wolbachia* were not present in any samples from Zambia (Fig. 2.9, Table 2.6). *M. rileyi* was present in 40% of samples from Lusaka (N = 15). The distribution of *M. rileyi* was significantly different between locations throughout Zambia ($\chi^2_6 = 23.88$, P=0.001).

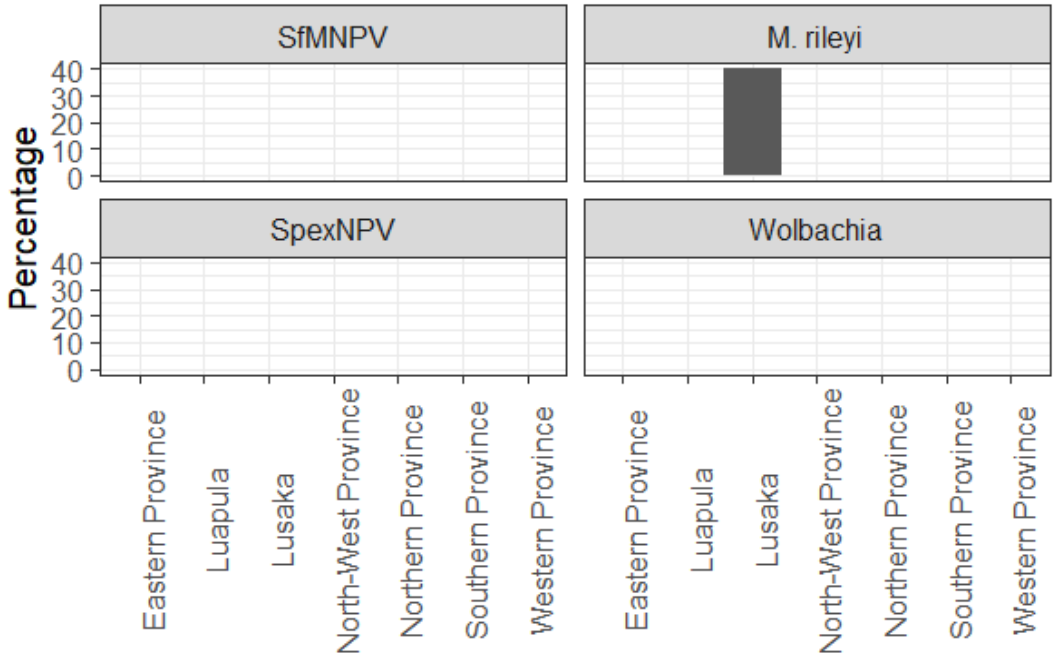


Figure 2.9 Percentage of larval samples from each location sampled in Zambia that tested positive for each microbe. The only microbial natural enemy identified in Zambia was *M. rileyi,* which was identified in only one location that larvae were collected from. Sample sizes are in Table 2.5.

Table 2.6 The prevalence of SfMNPV, SpexNPV, *M. rileyi* and *Wolbachia*. The table below shows the locations where larvae were collected and the number of larvae included in this study. Pathogen presence was detected by screening DNA extracted from larvae for the presence of microbial DNA, and the pathogen was recorded as either present or absent. Where possible, a binomial glm was carried out to determine if the prevalence of each pathogen significantly varied between locations within each country. Significant *P* values are in bold.

Country	**Location**	**N**	**Pathogen presence (%)**				**SfMNPV**			**SpexNPV**			***Wolbachia***			***M. rileyi***		
			SfMNPV	**SpexNPV**	***Wolbachia***	***M. rileyi***	**χ^2**	**df**	***p***	**χ^2**	**df**	***p***	**χ^2**	**df**	***p***	**χ^2**	**df**	***p***
Ghana	Twifo Ayaase	30	16.67	0	0	0	12.77	2	**0.002**									
	Aviation farm	4	100	0	0	0												
	UCC	38	34.21	0	0	0												
	All	72	30.56	0	0	0												
Kenya	Homa Bay	30	0	0	3	0							0.53	1	0.47			
	Embu	9	0	0	0	0												
	All locations	39	0	0	2.56	0												
Malawi	Bvumbwe	40	23	13	0	3	14.04	4	**0.007**	29.52	4	**>0.001**	3.56	4	0.47	5.32	4	0.256
	Lilongwe	31	32	23	3	0												
	Nchalo	40	3	30	0	5												
	Salima	30	20	0	0	0												
	Thyolo	40	15	0	0	0												
	All locations	181	17.68	13.26	0.55	1.66												
Rwanda	Ruhango	10	0	0	0	0	7.57	12	0.819	16.19	12	0.183	5.18	12	0.952	10.56	12	0.567
	Nyanza	9	0	0	0	0												
	Gisagara	10	0	0	0	0												
	Muhanga	8	0	0	0	0												
	Kirehe	10	0	0	10	0												
	Gatsibo	10	0	0	0	20												
	Kayonza	10	10	0	0	0												
	Nyagatare	10	10	0	0	0												
	Rusizi	10	0	30	0	0												
	Nyamasheke	10	0	0	0	0												
	Karongi	10	0	0	0	0												
	Ngororero	10	0	0	0	0												
	Kamonyi	10	0	0	0	0												

	All locations	127	1.57	2.36	0.79	1.57						
Sudan	Al Qadarif	28	10.71	0	3.57	0						
Zambia	Lusaka	15	0	0	0	40				23.88	6	**0.001**
	Western province	3	0	0	0	0						
	Eastern province	4	0	0	0	0						
	Southern province	3	0	0	0	0						
	Northern province	10	0	0	0	0						
	North-West province	7	0	0	0	0						
	Luapula	9	0	0	0	0						
	All locations	51	0	0	0	11.76						

2.4.3 The prevalence of SfMNPV, M. rileyi and SpexNPV varies significantly between countries, however, Wolbachia is at equally low prevalence across Africa

SfMNPV was the most prevalent natural enemy in Africa and its prevalence significantly varied between the six countries, with the mean percentage of SfMNPV-positive samples varying from 0 to 31% ($\chi^2_5 = 34.32$, *P*<0.001, Fig. 2.10 and Fig. 2.11). *M. rileyi* prevalence varied significantly between the countries, ranging from 0 to 12% of samples ($\chi^2_5 = 17.95$, P = 0.003, Fig. 2.9), as did SpexNPV, which ranged from 0 to 13% ($\chi^2_5 = 39.821$, P<0.001, Fig. 2.10). *Wolbachia*, however, did not significantly vary between the six countries, ranging from 0 to 4% ($\chi^2_5 = 4.596$, P = 0.467, Fig. 2.10).

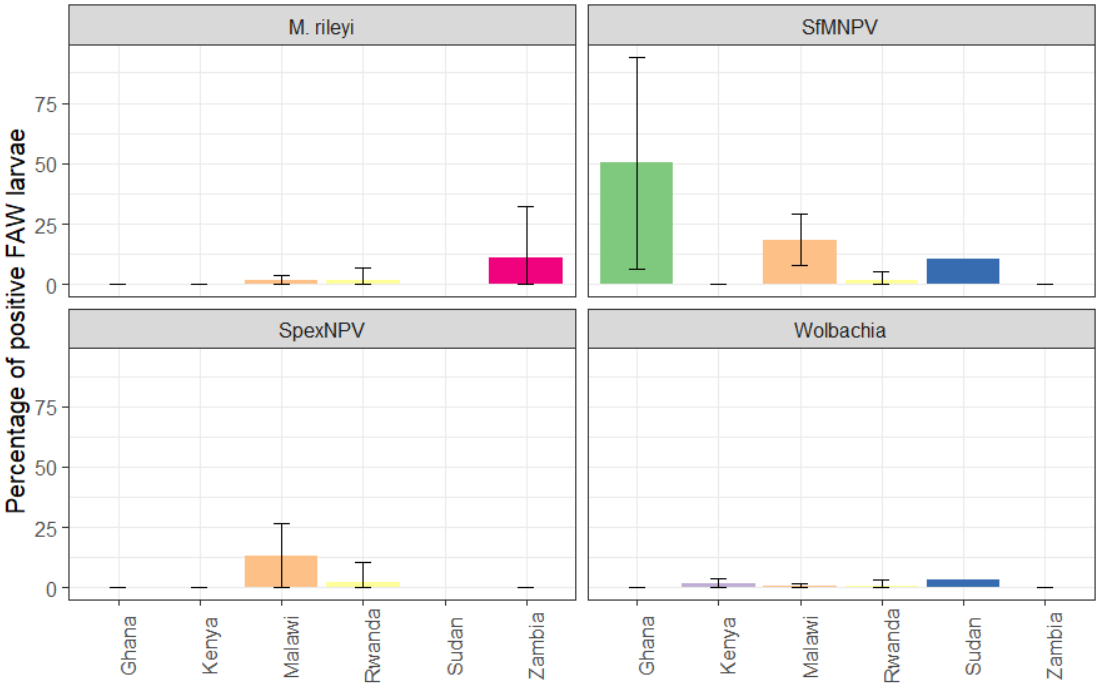


Figure 2.10: The mean (± standard deviation) percentage of FAW larvae with each microbe in each country. Pathogen presence was detected by screening DNA extracted from larvae for the presence of microbial DNA, and the pathogen was recorded as either present or absent. Sample sizes are in Table 2.6.

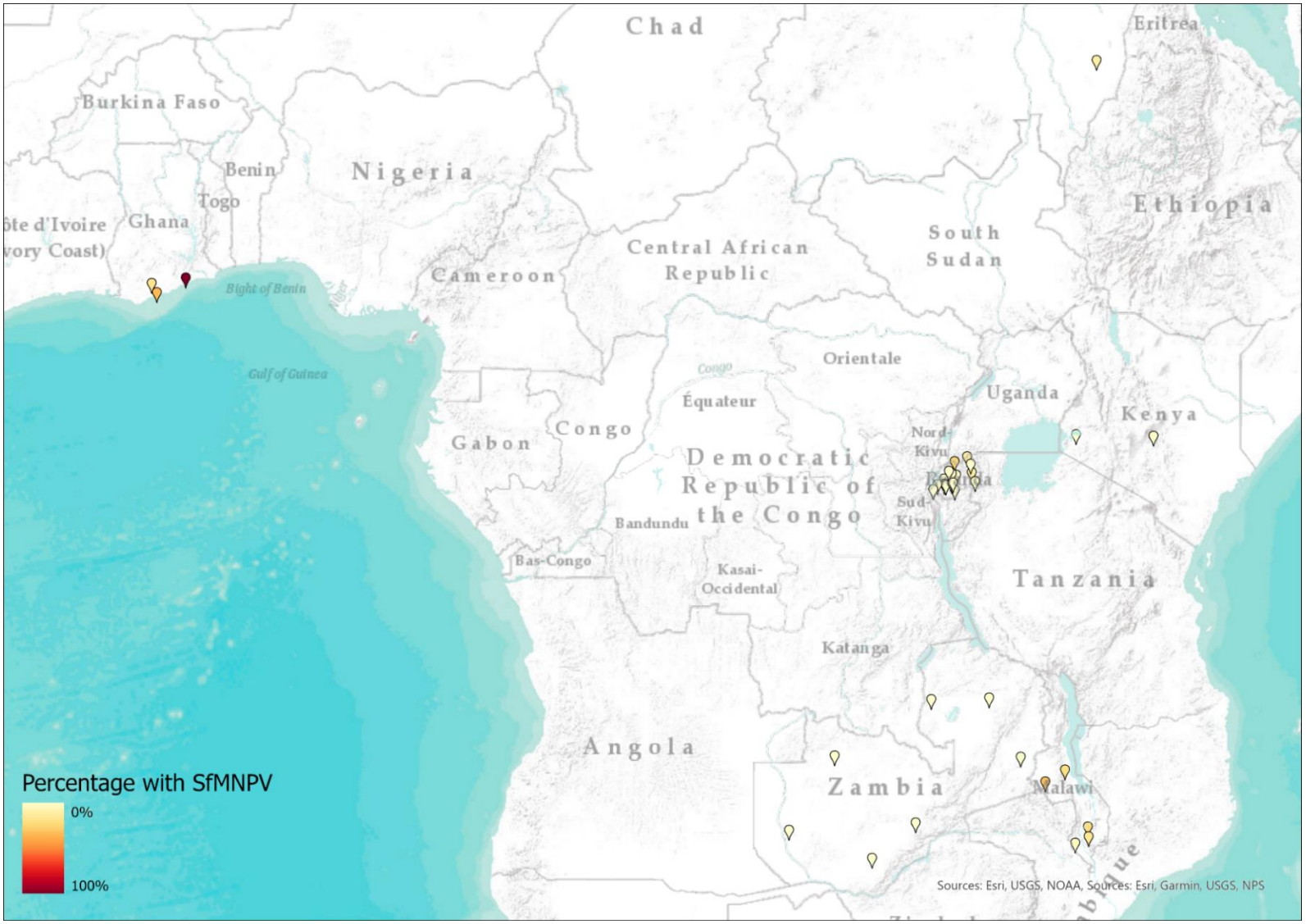


Figure 2.11 The percentage of FAW with SfMNPV found at each sampling site in Africa. Lighter colour represents low prevalence and darker colours represent high prevalence.

2.4.4 The impact of SpexNPV infection in FAW

SpexNPV did not cause mortality in FAW at any dose tested in this study (Table 2.7). There was some evidence that SpexNPV caused stunted growth in FAW (Fig. 2.12), however, this effect was not significant ($F_{1,5} = 0.13$, $P = 0.735$).

Table 2.7 The effect of different doses of SpexNPV on FAW larvae. Following exposure to SpexNPV larvae were monitored daily for mortality and development. Growth was recorded as stunted for individuals that were two or more instars smaller than the rest of the larvae throughout the experiment.

SpexNPV dose (OB / ml)	Number of larvae	Proportion dead	Proportion stunted
0	13	0	0.15
$1x10^2$	25	0	0.16
$1x10^3$	17	0	0.06
$1x10^4$	24	0	0.04
$1x10^5$	31	0	0.03
$1x10^6$	34	0	0.12
$1x10^7$	23	0	0.26

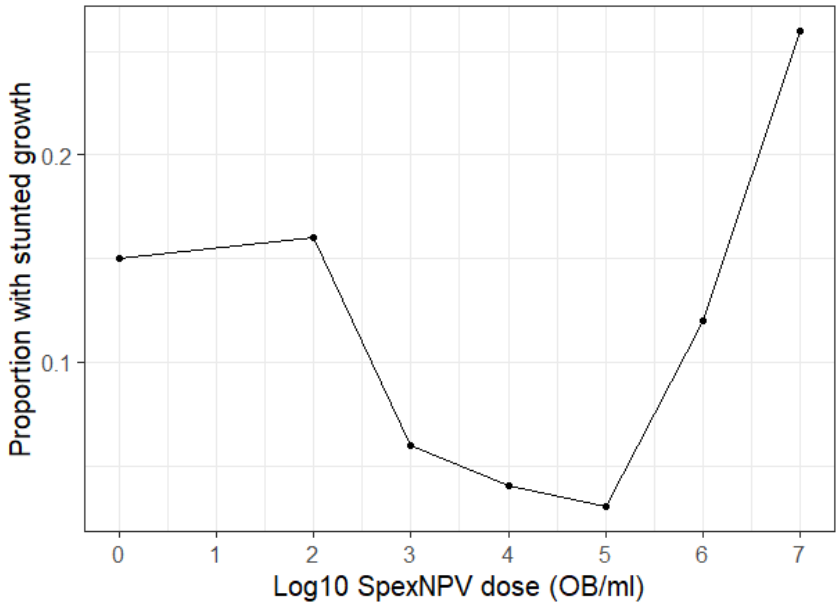


Figure 2.12 Development of FAW larvae infected with different doses (Log10 OB/ml) of SpexNPV. Growth was recorded as stunted for individuals that were two or more instars smaller than the rest of the larvae throughout the experiment.

2.4.5 The effect of environmental variables on SfMNPV distribution in Africa

2.4.5.1 Variation in meteorological, geographical, and temporal variables between countries and SfMNPV prevalence

The full principal component (PC) details, including loadings, contribution, and variance explained are shown in Table 2.8. PC1 explained 59% of variance and was based predominantly on temperature, rainfall and elevation suggesting that these have a strong influence on variation between the countries. PC2 was influenced strongly by time since FAW was first recorded in the country and days into the growing season, and this explained a further 18% of the variance.

Based on the PCs, the three most similar countries were Ghana, Malawi, and Kenya, mostly due to similarities in temperature and how long FAW had been in the country prior to when the larvae samples were collected (Fig. 2.13, Table 2.4). Rwanda had a stronger association with higher elevation whilst samples from Zambia were associated with sample collections later in the growing season (Fig. 2.13, Table 2.4). This highlights differences between sampling locations and the timing of sample collections, and this would add bias to models that include country when looking at SfMNPV prevalence, therefore PCs were used instead.

Using the PCs, SfMNPV prevalence (measured as % of positive samples) was predicted and showed that it was most closely related to temperature and days since FAW had first been recorded in the country, suggesting that these are important factors influencing the prevalence of SfMNPV (Fig. 2.13). PC1 and PC2 both significantly affected the distribution of SfMNPV (Z=-4, $P<0.001$ and Z=-2.91, P=0.004 respectively), suggesting that both geographical and temporal variables play an important role in determining SfMNPV prevalence in wild FAW populations across Africa. PC3 did not explain significant variation in the prevalence of SfMNPV (Z=-0.07, $P=0.95$), and a model comparison showed no significant difference between the model containing PC1 and PC2 and the model containing PC1, PC2 and PC3 ($\chi^2_{27} = 72.49$, $P=0.95$).

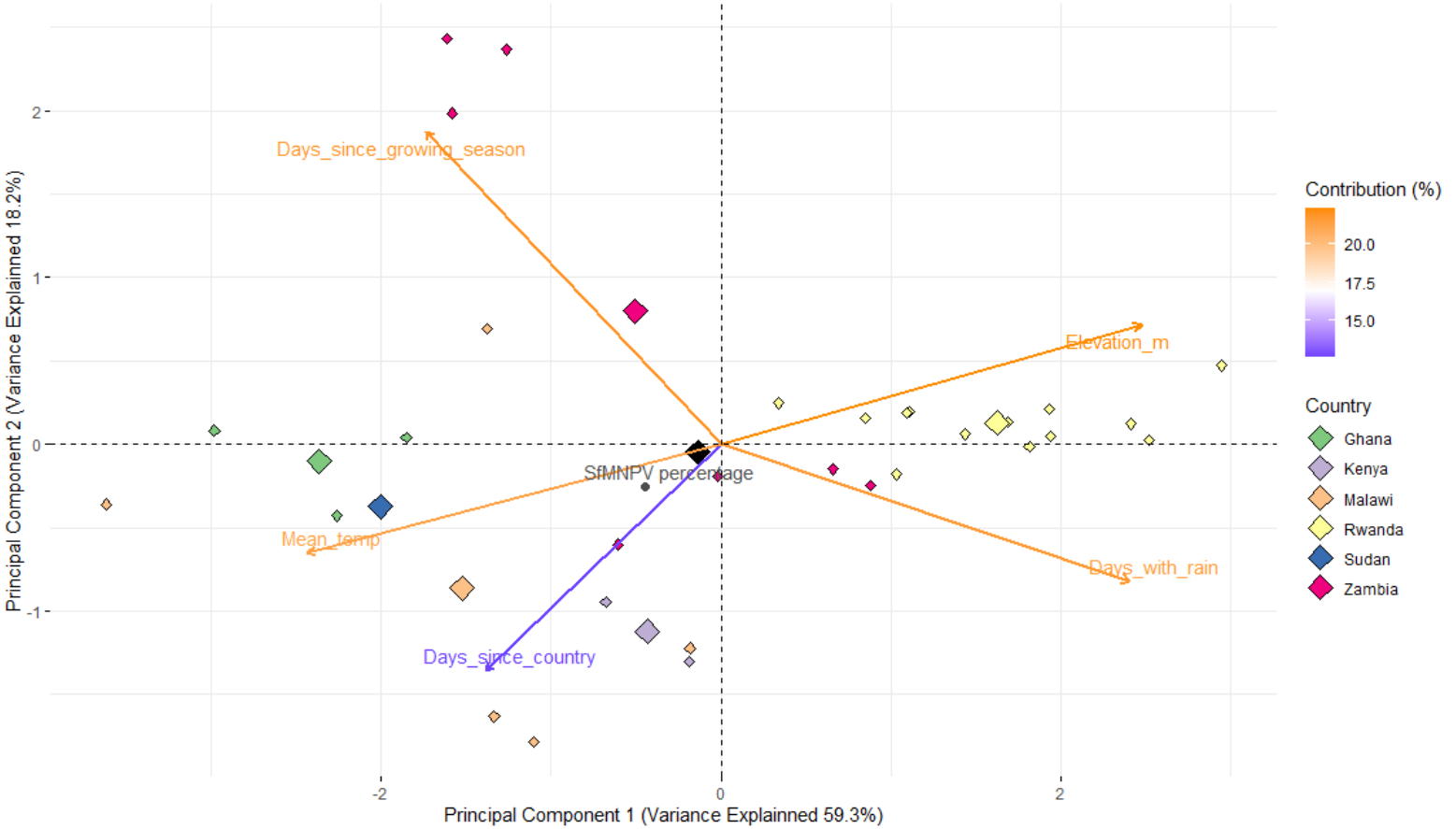


Figure 2.13 Principal components analysis (PCA) biplot. The PCA was conducted on the five environmental variables (*days_with_rain, mean_temp, elevation_m, days_since_country, days_since_growing_season*) included in this study. The plot is composed of the first two principal components (PC) plotted against each other. The colour of the arrows shows their contribution to the PCs (orange is higher contribution, blue is lower contribution) and the direction shows the correlation with other variables. The six countries are then plotted on to the biplot to show how country variations correspond to the environmental variables studied (e.g., sampling locations in Rwanda vary most due to elevation whereas most variation in Zambia is caused by when the samples were collected in the growing season). Smaller diamonds represent individual sample sites within countries, while the larger diamonds show the mean for that country. The overall mean (across countries) is shown by the large black diamond positioned towards the centre. The prevalence of SfMNPV was predicted based on the PCs and the position of this is shown by the label and the grey circle.

Table 2.8 The results of the principal components analysis on the five variables. The table shows the loading (L) and contribution (C) of each of the principal components (PC). The PC with an eigenvalue >1 are shaded in grey. The variables with the greatest loadings for each PC (>0.3 or <-0.3) are shown in bold.

	PC1		PC2		PC3		PC4		PC5	
	L	C	L	C	L	C	L	C	L	C
Days_since_growing_season	**-0.36**	13.26	**0.71**	50.38	**0.22**	11.08	**-0.47**	22.51	0.17	2.77
Days_since_country	-0.28	8.49	**-0.51**	26.48	**0.80**	64.24	0.04	0.19	0.08	0.69
Elevation_m	**0.52**	26.94	0.27	7.33	0.27	7.42	**0.42**	17.74	**0.64**	40.56
Days_with_rain	**0.50**	25.38	-**0.31**	9.71	<0.01	<0.01	**-0.77**	59.52	0.23	5.38
Mean_temp	**-0.51**	26.02	-0.25	6.09	-0.42	17.25	-0.02	0.04	0.71	50.60
Standard deviation	1.72		0.95		0.89		0.51		0.27	
Variance explained	59.29		18.19		15.85		5.22		1.44	
Cumulative variance	59.29		77.48		93.34		98.55		100	

2.4.5.2 The impact of geographic and temporal variables on SfMNPV prevalence across Africa

Considering the high variation between countries shown in the PCA, but the strong effect of both geographical (PC1) and temporal (PC2) variables, SfMNPV prevalence was modelled based on these variables alone (country excluded) to give an idea of their impact on SfMNPV prevalence. The most variation in SfMNPV prevalence was explained by a model including three variables: mean temperature (*Mean_temp*), days since growing season (*Days_since_growing_season*) and elevation (*Elevation_m*) (Table 2.9).

Lower temperatures and lower elevation were significantly associated with a higher prevalence of SfMNPV throughout Africa (m9: Est.= -0.305±0.11, Z=-2.708, *P*=0.008, Est.=-0.003±0.001, Z=-4.708, *P*<0.001 respectively). In contrast, samples collected later in the growing season, led to a lower prevalence of SfMNPV and including this term in the model led to lower AICc suggesting that time into the growing season explained more variation (Table 2.9). However, the effect of growing season in the model with the lowest AICc was not statistically significant (m9: Est.= -0.012±0.007, Z=-1.85, *P*=0.064).

Table 2.9 Comparisons of the seventeen different models for Africa. A variety of binomial GLMS were carried out to determine the effects of the five environmental variables on the prevalence of SfMNPV in Africa, and models were compared using MuMin in R. AICc is the Akaike information criterion corrected for small sample sizes. Delta is the difference in AICc between the model and the top model. Models with delta <2 are of equal significance. The weight indicates the relative likelihood of the model compared to the other models. Due to small sample size no interaction terms were included in the models as this led to over parametrising the data. Pseudo R^2 was calculated using r.squaredLR function in MuMin package, based on the Nagelkerke method.

	Days with rain	Mean temp	Elevation (m)	Days since country	Days since growing season	pseudo R^2	df	logLik	AICc	delta	weight
m9		-0.305	-0.003		-0.012	0.73	4	-45.19	99.93	0.00	0.67
m1	-0.011	-0.365	-0.004		-0.016	0.73	5	-44.94	102.27	2.35	0.21
m5			-0.001			0.62	2	-50.21	104.85	4.92	0.06
m10			-0.002		-0.008	0.64	3	-49.32	105.53	5.60	0.04
m11	0.016		-0.002		-0.004	0.66	4	-48.40	106.35	6.42	0.03
m16		0.165		0.001		0.50	3	-54.35	116.19	15.64	0.00
m13	0.030	0.229		0.001	0.010	0.57	5	-51.90	117.95	16.27	0.00
m14		0.156		0.001	0.003	0.50	4	-54.21	117.53	18.02	0.00
m3		0.159				0.35	2	-58.25	120.94	21.01	0.00
m7	0.016	0.217				0.39	3	-57.33	121.55	21.63	0.00
m8	0.025	0.224			0.007	0.42	4	-56.44	122.42	22.49	0.00
m12		0.152			0.002	0.35	3	-58.14	123.17	23.24	0.00
m6				0.001		0.25	2	-60.51	125.45	25.52	0.00
m15				0.001	0.007	0.30	3	-59.31	125.51	25.59	0.00
m17	0.005			0.001	0.008	0.31	4	-59.21	127.96	28.03	0.00
m4					0.006	0.07	2	-63.65	131.73	31.80	0.00
m0						0.00	1	-64.80	131.74	31.81	0.00
m2	-0.010					0.05	2	-64.05	132.52	32.59	0.00

2.4.5.3 Influence of geographical and temporal variables in Malawi

The PCA showed strong differences between the six countries, therefore, to better understand the impact of each variable independently on SfMNPV distribution, Malawi was modelled as a case study as Malawi was the only country for which there were sufficient data (N=181, >3 locations with SfMNPV). Fifteen models were compared using *MuMin* and the three models that can be considered equal due to delta <2 are discussed here (all fifteen models are shown in Table 2.10).

The model that explained the most variation in SfMNPV prevalence in Malawi suggested that rainfall (*Days_with_rain*) alone had the greatest impact (Table 2.10), with the log-likelihood of SfMNPV being present in FAW increasing by 0.04±0.01 for each extra day with rain (m1M: Est.= 0.04±0.01, Z=2.82, *P*=0.008, Fig. 2.14A).

The next best model included only the duration that FAW has been present in the country (*Days_since_country*), which suggests that the prevalence of SfMNPV has increased the longer FAW have been in Malawi (m4M: Est.= 0.01±0.003, Z=2.70, *P*=0.007, Fig. 2.14B).

The third model that should be considered is the period in the growing season that the samples were collected (*Days_since_growing_season*), which suggests that SfMNPV prevalence in FAW declines the further into the maize growing season; the more developed the crop the less likely FAW are to have SfMNPV (m5M: Est.= -0.02±0.01, Z=-2.61, *P*=0.009, Fig. 2.14C).

Based on AICc, all other models explained less variance than the intercept only model (Table 2.10), therefore this suggests that temperature (*Mean_temp*) and elevation (*Elevation_m*) do not significantly influence the prevalence of SfMNPV in FAW in Malawi.

Table 2.10 Comparisons of the fifteen different models for Malawi. A variety of binomial GLMS were carried out to determine the effects of the five environmental variables on the prevalence of SfMNPV in Malawi, and the models were compared using MuMin in R. AICc is the Akaike information criterion corrected for small sample sizes. Delta is the difference in AICc between the model and the top model. Models with delta <2 are of equal significance. The weight indicates the relative likelihood of the model compared to the other models. Due to small sample size, models were limited to two variables and no interaction terms were included in the models as this led to over parametrising the data. Pseudo R^2 was calculated using r.squaredLR function in MuMin package, based on the Nagelkerke method.

Model	Days with rain	Mean temp	Elevation (m)	Days since country	Days since growing season	Pseudo R^2	df	logLik	AICc	Delta	weight
m1M	0.04					0.85	2	-10.59	31.18	0	0.39
m4M				0.01		0.82	2	-11.04	32.08	0.9	0.25
m5M					-0.02	0.79	2	-11.44	32.88	1.7	0.17
m0M						0.00	1	-15.26	33.85	2.67	0.1
m3M			0			0.64	2	-12.71	35.42	4.24	0.05
m2M		-0.16				0.60	2	-12.99	35.98	4.8	0.04
m11M		-0.24		0.01		0.94	3	-8.27	46.53	15.35	0
m6M	0.04	-0.21				0.94	3	-8.32	46.65	15.46	0
m14M			0		-0.03	0.94	3	-8.36	46.72	15.54	0
m12M		-0.25			-0.03	0.94	3	-8.4	46.79	15.61	0
m13M			0	0.01		0.93	3	-8.57	47.15	15.96	0
m7M	0.03		0			0.93	3	-8.62	47.24	16.06	0
m8M	0.19			-0.04		0.89	3	-9.7	49.39	18.21	0
m9M	0.07				0.03	0.87	3	-10.26	50.52	19.34	0
m15M				0.01	0.01	0.82	3	-10.99	51.98	20.79	0
m10M		0.12	0			0.65	3	-12.66	55.31	24.13	0

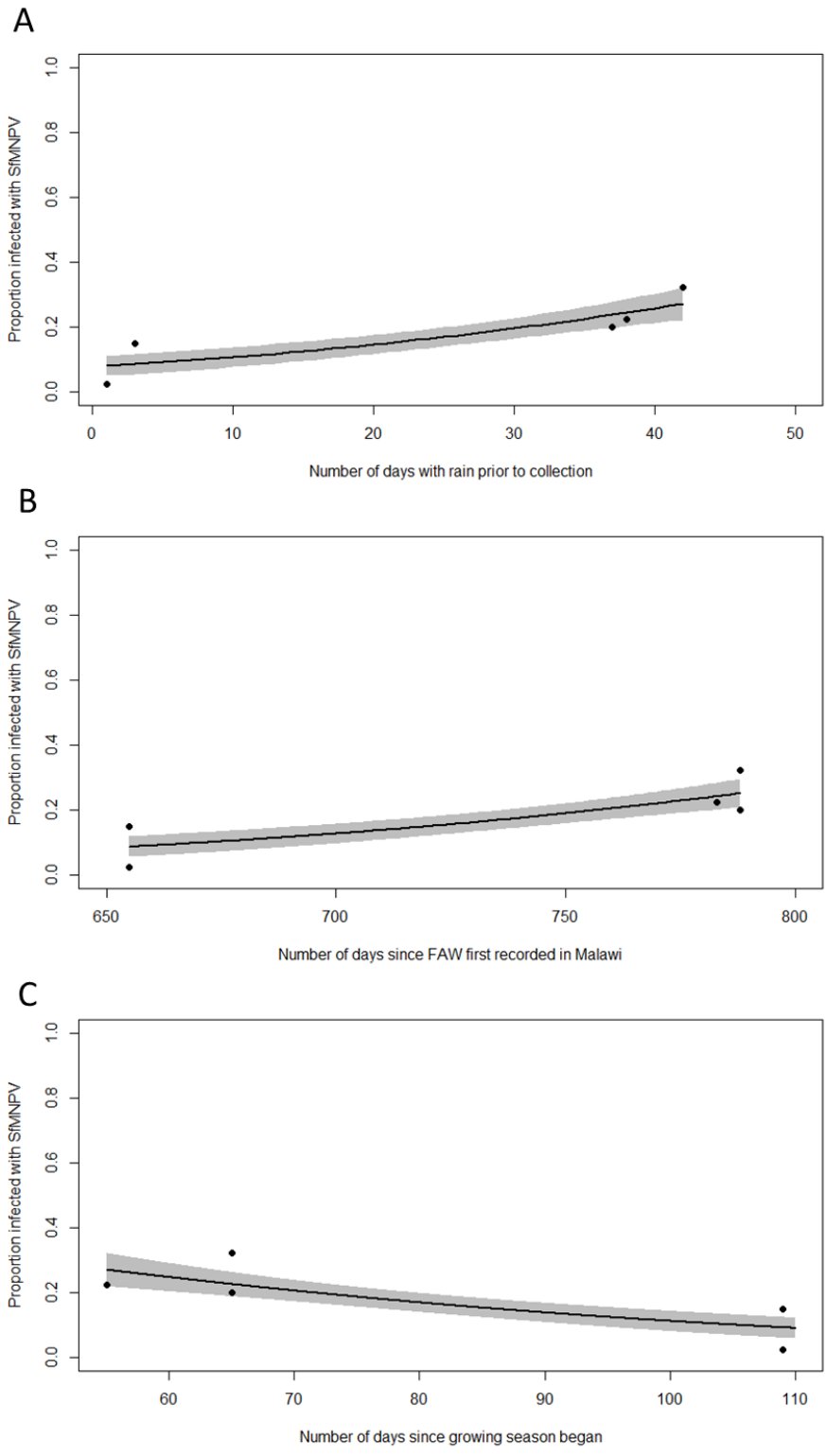


Figure 2.14 The effect of different variables on SfMNPV prevalence in FAW in Malawi. The binomial GLMs that explained the most variation in the prevalence of SfMNPV were selected and prevalence was modelled to give an indication of their effects, though it should be remembered that due to the significant correlation between variables it is unlikely that any of these variables would have an impact alone. The figures shows A) The prevalence of SfMNPV and the number of days with rain in the development days prior to when FAW were sampled. B) The prevalence of SfMNPV and the number of days since FAW was first recorded in Malawi. C) The prevalence of SfMNPV and the number of days into the growing season when FAW were sampled. The shaded areas show the 95% confidence interval, and the line shows the predicted prevalence of SfMNPV.

2.5 Discussion

This study presents evidence that at least four different microbial natural enemies are present within African populations of FAW, with at least one present in each of the six countries we sampled. Two natural enemies (SfMNPV and *M. rileyi*) identified were hypothesised to be present based on their prevalence in native and other invasive populations, however, two natural enemies (SpexNPV and *Wolbachia*) were present unexpectedly as neither had previously been recorded in either native or invasive FAW. The prevalence of natural enemies varied significantly between the six countries, as well as within countries for some pathogens. Here, for each pathogen, factors that could explain the observed pattern in prevalence are discussed, as well as potential impacts of the presence of infections in FAW in Africa.

2.5.1 SfMNPV is the most common covert infection in FAW in Africa, and its prevalence is influenced by weather, the time since FAW invasion and the time into the crop growing season.

The most prevalent microbial natural enemy was SfMNPV. FAW had not been recorded in Africa before 2016 so it is very unlikely that SfMNPV occurred naturally in Africa prior to this invasion. It occurs naturally within the Americas, with isolates identified in many countries including Mexico and Colombia at varying prevalence (Gómez et al., 2013; Cipriano et al., 2013; García-Banderas et al., 2020), therefore it is likely that some of the first FAW in Africa carried the virus over to Africa. Further supporting this theory, an SfMNPV isolate from FAW in Nigeria was most similar to another naturally occurring SfMNPV isolate collected in Brazil (Wennmann et al., 2021).

The higher prevalence of SfMNPV compared to the other microbes studied meant that factors potentially affecting its distribution could be studied in more detail, and it was found that it was associated with a mix of both meteorological, temporal, and geographical variables.

As hypothesised, the time since FAW had first been recorded in the country was shown to be linked to SfMNPV prevalence both in the PCA analysis and within Malawi. This relationship has been observed between NPV prevalence and another invasive moth, the gypsy moth (*Lymantria dispar*), in North America, with the virus increasing over time in the spreading populations (Hajek and Tobin, 2011). Invasive populations initially benefit from fewer natural enemies than in their native range meaning they are able to rapidly spread, this is known as the enemy release hypothesis (Cornell and Hawkins, 1993; Torchin and

Mitchell, 2004). Therefore, this study provides evidence that the FAW may have undergone this process and escaped some natural enemies for a period of time when first reaching certain countries, which could have been a factor in their rapid spread throughout Africa, and into Asia and Australasia in recent years. However, time since invasion was not considered to be important in models when looking at Africa as a whole suggesting some country variation might be affecting this relationship.

Time into growing season was a key factor in explaining the prevalence of SfMNPV. However, the observed pattern led to the rejection of the hypothesis that SfMNPV prevalence would increase later in the growing season with a decrease in prevalence observed across Africa as a whole and in Malawi. Later into the growing season the population density of FAW reduces (Caniço et al., 2020). This is because FAW larvae are solitary and cannibalistic, meaning that on mature plants there is usually only one larvae present. NPV persist at higher levels in larger populations, such as SpexNPV in *S. exempta*, and this is reflected as a evolutionary feature by higher immunity at higher population density suggesting this is a well established pattern (Reeson et al., 1998; Vilaplana et al., 2008). Therefore, the lower population densities observed in FAW later in the growing season are likely to have reduced transmission of SfMNPV with fewer chances for horizontal transmission with larvae rarely coming into contact with viral-killed cadavars. In addition, time into growing season could be a factor in prevalence as plant defences are stronger in mature corn plants, and these defenses can influence the effect of infection. For example, SfMNPV efficacy was lower and mortality reduced in FAW if the crop they were feeding on was constitutively expressing jasmonic acid (JA) (Shikano et al., 2017b; Shikano et al., 2017a).

Generally, models incidcated that SfMNPV prevalence was higher when temperatures were lower and when more days experienced rainfall. Research has been carried out on the effects of weather variables on SfMNPV used for biocontrol (Fuxa and Richter, 2001), however, this is the first study to consider the effects of it on naturally occuring SfMNPV. This study provides evidence that the effects may be quite similar in sprayed and naturally occuring SfMNPV because previous work showed SfMNPV occulsion bodies were able to spread more after precipitation (Fuxa and Richter, 2001). In line with the hypothesis, this study found an increase in rainfall led to higher prevelance of SfMNPV suggesting increased transmission may have occured. Additionally, increases in rainfall are often associated with insect outbreaks, meaning population densities might be higher, such as in *S. exempta* (Harvey and Mallya, 1995). However, due to limitations in data collections

these differences could be due to geographical variation and further investigation is needed to clarify the effects of rainfall on SfMNPV prevalence.

Temperature did not have a significant impact on SfMNPV prevalence in Malawi, however, it did across Africa as a whole. It was hypothesised that higher temperatures would lead to higher prevalence of SfMNPV due to increases in FAW abundance in warmer weather, however, this hypothesis was rejected because SfMNPV prevalence declined as temperatures increased. All temperatures recorded in this study were within the survival range for NPV, though prolonged exposure at the higher end of recorded temperatures (around 30°C) could lead to degradation (Ignoffo, 1992). Additionally, at higher temperatures NPV is more likely to be exposed to variables that can have negative impacts on OB survival such as increased exposure to UV light (Ignoffo et al., 1977; Ignoffo, 1992). Contradictory to our results, previous work carried out on NPV in the invasive gypsy moth in North America found a significant increase in viral prevalence as temperature increased in April and June (Hajek and Tobin, 2011). However, the range of temperatures during the study period were considerably different between Hajek and Tobin (2011) and this one, with the range of temperatures being 3 to 21°C in North America and 16 to 28°C in Africa. This highlights the need for research to be carried out on FAW and their natural enemies as populations spread throughout the world as the effects of variables, such as temperature, may not be generalisable between locations.

Elevation was an important factor in predicting SfMNPV prevalence for Africa, and interestingly no sampling locations with elevation >1000m had SfMNPV present. However, it is hard to determine whether this a direct effect of elevation or an interaction with other factors. One explanation is that it could be an effect of other variables such as weather, as elevation was strongly correlated to temperature, and both showed a significant relationship with SfMNPV in Africa, with lower temperatures and elevation resulting in higher prevalence of the virus. Furthermore, behavioural factors such as FAW movements and overwintering locations could be influenced by elevation in Africa (Cokola et al., 2020). Therefore, this would be reflected in SfMNPV transmission and prevalence through how FAW spreads and how populations mix.

Overall, these results show that SfMNPV prevalence varies considerably due to environmental and temporal factors. Whilst the effects of indivual variables on SfMNPV prevalence have been discussed here, it is important to remember that these variables are correlated and inextricably linked. Therefore no one variable acts alone to influence SfMNPV

prevalence, and it is a combination of a multitude of factors that leads to the within, and between, country variantion observed.

2.5.2 SpexNPV is present in FAW in Malawi and Rwanda, providing the first evidence of natural species spillover from African armyworm.

This study provided the first evidence of SpexNPV crossing over into FAW populations naturally, with SpexNPV found in FAW in Malawi and Rwanda. NPVs range from monospecific to generalist pathogens, with a wide range of factors affecting their host range, including life history traits of their target host (Lepidoptera) and genetic variation in the viruses themselves (Goulson, 2003; Thiem and Cheng, 2009).

Before this study, there was no record of SpexNPV infecting any other host species apart from its known target (*S. exempta*) in the lab or field. Prevalence of SpexNPV can be very high in its native host *S. exempta,* with 97% of field collected adults testing positive, with 21% showing overt signs of infection (Graham et al., 2012), so it is likely that FAW picked up this infection naturally within the field. SpexNPV would not have naturally been in contact with FAW before, therefore this species spillover happened relatively quickly. The impact of SpexNPV on FAW is not yet understood. The laboratory bioassay carried out in this study with SpexNPV in FAW found no mortality occurred and it was not possible to determine whether the virus was actively replicating in its hosts. However, there was some evidence of stunted growth in SpexNPV-challenged FAW. Therefore, SpexNPV may only cause covert or latent infections and have no impact on mortality in the field, but it could affect the development of FAW.

Co-infections of both SpexNPV and SfMNPV were observed in both these countries. Little research has been carried out on the impact NPV co-infections, however, there is some evidence from an *in vitro* study that NPV from different target hosts could help each other replicate, such as *Bombyx mori NPV* (BmNPV) and *Autographa californica NPV* (Kondo and Maeda, 1991). However, other viral co-infections prevented replication of each other, such as BmNPV and *Spodoptera litura NPV* (SlNPV) (Kondo and Maeda, 1991; Shirata et al., 2004). It is important to note that co-infection *in vitro* could lead to the formation of recombinant NPV with a wider host range that can cause mortality in their host (Kondo and Maeda, 1991). There is also evidence of recombinant NPV occurring naturally between SfMNPV and SlNPV in FAW in the Americas, therefore this is not the first time non-target NPV has been recorded in FAW when the target host *Spodoptera* overlap on the same crop (Barrera et al., 2015).

2.5.3 M. rileyi is rare but present in Malawi, Rwanda and Zambia, and causes both overt and covert infections.

This study confirmed that the fungal pathogen *M. rileyi* was present in samples collected in Africa, and able to cause both covert and overt infections. Though present, *M. rileyi* was rare in this study and was only identified in Malawi, Rwanda and Zambia. Similarly, another study looking at FAW gut microbiomes from Kenya identified *M. rileyi* in just one of eighteen samples sequenced (Gichuhi et al., 2020). The highest prevalence of *M. rileyi* in FAW in corn fields in India occurred in the middle of the growing season when humidity was the highest due to high temperatures following a period of heavy rainfall (Visalakshi et al., 2020). Therefore, as most samples in this study were collected at the start or end of the growing season in Africa this pattern of *M. rileyi* infection could have led to the low prevalence of *M. rileyi* recorded here. Additionally, this study focused on covert infection so samples screened had no obvious signs of infection and, therefore, cadavars with signs of mortality caused by fungus would not have been collected.

Though *M. rileyi* appears to be rare in Africa, its presence does need to be considered. No co-infections with *M. rileyi* and other microbes screened were identified. Determining how FAW respond to co-infections is important as a coinfection of SfMNPV and *M. rileyi* can increase mortality when both pathogens are able to infect the host simultaneously (Souza et al., 2019).

Due to the rarity of reported cases of *M. rileyi* in native populations of FAW (Ignoffo and Garcia, 1985; Fronza et al., 2017; Álvarez et al., 2018), it is likely that the source of these infections in FAW in Africa is spillover from closely related species, such as *S. exempta* (Rose et al., 2000).

2.5.4 Wolbachia has low prevalence in FAW across Africa.

Wolbachia was found to be present at low prevalence in four of the six countries, suggesting that it is widespread. The effect of *Wolbachia* on SfMNPV infection should be considered as it could change the response of FAW to infection. *Wolbachia* has not previously been recorded in FAW, and it was not found to be present in a lab strain originally collected from Florida (Dumas et al., 2015). However, multiple studies have shown that *Wolbachia* is capable of infecting and surviving in FAW cells (Sf9 cell line) (Herbert and McGraw, 2018; Furukawa et al., 2008). This suggests that *Wolbachia* would be able to infect FAW larvae should it be exposed to it. It is likely that FAW were infected with *Wolbachia* whilst in Africa due to the absence of *Wolbachia* in FAW in the Americas alongside the very

low prevalence of *Wolbachia* in South America where it is thought the invasive FAW originated from (Ahmed et al., 2015a; Dumas et al., 2015; Nagoshi et al., 2018b).

One theory proposed here is that a parasitoid vector may have caused the spread of *Wolbachia* from *S. exempta* to FAW, and that it has since persisted in FAW populations through vertical transmission. It has been shown that *Wolbachia* can survive in the mouthparts of a parasitoid vector and be transmitted to other hosts for around 48 hours (Ahmed et al., 2015b). Considering that co-infection with SpexNPV and SfMNPV was observed in this study, it suggests that FAW and *S. exempta* have been in close contact since FAW first invaded Africa. Furthermore, it has previously been confirmed that *Wolbachia* is present in *S. exempta* in Africa at high prevalence (56%), and was strongly associated with SpexNPV by increasing the susceptibility of *S. exempta* to the virus (Graham et al., 2012), thus increasing the likelihood that a parasitoid may have picked up *Wolbachia* and horizontally passed it on to FAW.

2.5.5 Conclusion

This study revealed four different microbial natural enemies are present within FAW in Africa. These could help control populations naturally and affect the efficacy of biocontrol treatments and open the door for the development of biopesticides. If any of these microbial natural enemies could be extracted from FAW in Africa it may be possible to culture them to determine virulence and any potential for developing them for biopesticides.

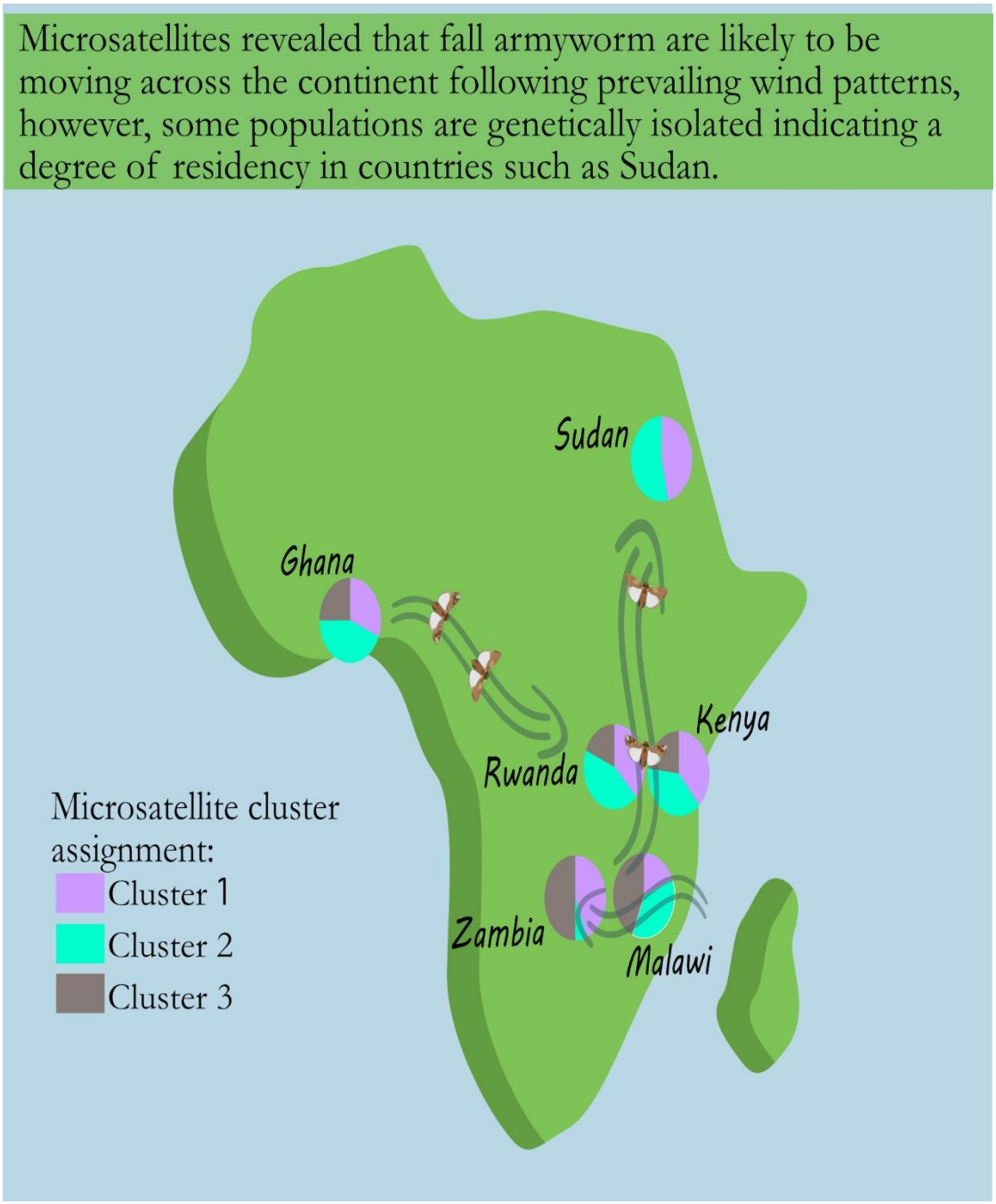
Unfortunately, as samples were originally collected for strain identification, they were stored in ethanol and therefore unsuitable for RNA extraction to determine viral activity, thus only presence or absence data from viral DNA could be collected. Whilst some FAW strain identification was trialled as part of this project initially (Chapter Three), multiple studies were published that showed inconsistencies with strain identification (Nagoshi et al., 2017b; Nagoshi et al., 2018b), and eventually it was determined that African FAW were a hybrid of both the recognised corn and rice strains (Zhang et al., 2020). However, it would be important for future work to determine transcriptionally active virus levels in field populations for both SfMNPV and SpexNPV.

As shown in Chapter Five and previous work, viral infections can significantly change flight ability of FAW (Pearson, 2016), therefore it is important that this relationship be better understood in FAW in Africa as it could lead to changes in population movements throughout the continent. For example, some areas could be at greater risk of FAW

infestations if lower prevalence of SfMNPV means FAW are more likely to travel further distances.

Overall, this study has addressed a large gap in the current understanding of natural enemies in FAW in Africa, and shown that SfMNPV, SpexNPV, *M. rileyi* and *Wolbachia* are all present throughout Africa at varying levels of prevalence. It has increased knowledge of the factors that influence the natural distribution of SfMNPV in Africa, showing the importance of weather, elevation and time in determining prevalence.

Chapter 3 - Fall armyworm populations frequently mix with others throughout Africa.



The results presented in this chapter have been published in Scientific Reports, a copy of the paper is included at the end of this thesis:

Withers, A. J., et al. (2021) Microsatellites reveal that genetic mixing commonly occurs between invasive fall armyworm populations in Africa. Scientific Reports. 11,20757. 10.1038/s41598-021-00298-3.

3.1 Abstract

Understanding the population structure and movements of invasive crop pests is extremely important. It can help to mitigate crop damage, and improve our understanding of high risk areas when predicting outbreaks or insecticide resistance. Determining population structure in the fall armyworm (FAW, *Spodoptera frugiperda*) in Africa has been a challenge due to genetic mutations affecting the traditionally used markers for strain identification and haplotypes; *mitochondrial cytochrome oxidase I* (COIB) and the Z-chromosome-linked *Triosephosphate isomerase* (Tpi) haplotypes. Here, traditional markers (COIB, TpiE4 and TpiI4) were used alongside highly variable repeat regions called microsatellites to compare what the two approaches can tell us about FAW population structure in Africa. Larvae were collected from six different countries (Ghana, Kenya, Malawi, Ghana, Sudan and Zambia) between 2017 and 2019. The COIB and TpiE4 markers showed high level of disagreement for strain identification, with the COIB suggesting most larvae were rice strain whereas the TpiE4 markers suggested they were corn. There was very limited genetic diversity using the COIB marker with only two haplotypes recorded, whereas using the TpiI4 marker there were six different haplotypes recorded. Based on the Tpi haplotypes there was very little evidence of genetic structuring between FAW populations across Africa, with most genetic variation occurring between individual larvae regardless of where they were collected. By contrast, there was much more genetic diversity identified using the microsatellite approach, enabling a more in-depth analysis of population structure. Similarly to the TpiI4 marker, the microsatellites revealed a largely panmictic population of FAW, however, there was some evidence of genetic structuring of FAW between countries. This genetic structuring largely follows known wind patterns, and it is hypothesised here that FAW are using their long-distance flight ability alongside utilising wind patterns to frequently move throughout Africa leading to frequent population mixing. These combined approaches provide important evidence that genetic mixing between FAW populations in Africa may be more common than previously reported. This has important consequences for FAW management when considering factors such as the spread of insecticide resistance and crop infestations across borders.

3.2 Introduction

The fall armyworm (FAW, *Spodoptera frugiperda*) is a highly invasive crop pest in Africa, Asia and Australasia (CABI, 2020). It is native to North America where it is largely migratory, surviving winters in southern Florida and Texas and then migrating north as the temperature warms, though there is some evidence that parts of South America, such as Puerto Rico, have limited interactions with FAW from elsewhere in America (Nagoshi and Meagher, 2008; Nagoshi et al., 2010; Westbrook et al., 2016). Its migratory nature means it has a strong flight ability, and some individuals can disperse as far as 300 miles before oviposition (Johnson, 1987). Wherever it disperses to, the effects can be devastating. In outbreak regions the fall armyworm can cause millions of tonnes of crops to be lost, resulting in huge economic losses as well as food shortages (Abrahams et al., 2017).

Understanding the migratory routes of FAW is important as these can be used to predict areas at risk and give farmers warning for early intervention techniques (Westbrook et al., 2016; Nagoshi et al., 2017a). Additionally, understanding gene flow can help to predict outbreaks and foresee the spread of insecticide resistance that primarily spreads through the mixing of populations which leads to resistance alleles becoming more common in populations that were previously susceptible (Nagoshi et al., 2017a; Arias et al., 2019).

There is currently a lot known about FAW population structure and movements in its native region (America), enabling farmers to deal with outbreaks and minimise losses (Nagoshi et al., 2007; Westbrook et al., 2016; Nagoshi et al., 2017a; Nagoshi et al., 2018a). Much less is known about potential migration and population mixing in Africa, and much of the available research has been based on *mitochondrial cytochrome oxidase I* (COIB) and the Z-chromosome-linked *Triosephosphate isomerase* (Tpi) haplotypes (Nagoshi et al., 2018a; Nagoshi et al., 2019a; Nagoshi et al., 2020). However, there is some disagreement between COIB and TPI haplotypes in FAW in Africa for strain identification, with evidence suggesting that the COIB haplotypes are less reliable in distinguishing invasive populations across Africa and Asia (Nagoshi et al., 2018a; Nagoshi et al., 2020; Nayyar et al., 2021). This disagreement means that most individuals are identified as the rice strain with the COIB marker, and the same individuals are then identified as the corn strain with the TpiE4 marker (Nagoshi et al., 2018a; Nagoshi et al., 2019a; Nagoshi et al., 2020). Given that the majority of samples have been collected on maize plants it has been suggested that the most accurate marker is likely to be the TpiE4 marker. This confusion with the COIB and TPI markers is most likely due to the hybridization of the corn and rice strains which has occurred in the invasive populations

since FAW left its native range (Zhang et al., 2020). Furthermore, the COIB and Tpi markers show very little variation in the invasive FAW populations, with only two COIB haplotypes (COIB-RS, CSh4) and three TpiI4 haplotypes (TpiCa1a, TpiCa2a, TpiCa2b) previously reported in South Africa and India (Nagoshi et al., 2019a; Nayyar et al., 2021).

There is strong evidence that the invasive populations in Asia are originally from Africa, with FAW from both continents showing similar haplotype frequencies and the same mutation affecting the COIB strain identification marker (Nagoshi et al., 2019a; Nagoshi et al., 2020; Nayyar et al., 2021). Understanding the source of the FAW population via population genetics approaches is, therefore, an important factor in curbing further spread as this can help control spreading populations of FAW by suggesting the likely origin which could provide information such as insecticide resistances. This study addresses this by using microsatellites to determine population structure and genetic diversity across Africa.

Microsatellites are highly variable, repeat regions of DNA that are useful when studying genetic mixing within insect populations at continental scales. For example, in two hoverfly species (*Episyrphus balteatus* and *Sphaerophoria scripta*) microsatellites revealed high levels of genetic mixing suggesting frequent migratory movements across Europe over a very large scale, predominately occurring along the North-South axis (Raymond et al., 2013). Additionally, microsatellites can be used to detect genetic differentiation at much smaller scales, such as between reduviid bugs (*Triatoma dimidiate*) in neighbouring villages in Guatemala (Stevens et al., 2015).

Microsatellites have previously been identified in FAW using populations from Texas, Mississippi, Puerto Rico and Brazil (Arias et al., 2011; Pavinato, 2013). These microsatellites were varied enough to distinguish between three genetically-distinct populations and were able to identify migrants between those populations (Pavinato, 2013). Therefore, considering the limited variability and confusion around the COIB and TPI haplotypes, FAW microsatellites might be a better way to identify population structure in FAW across Africa.

Furthermore, as microsatellites can pick up smaller-scale differentiation. Given the context of this thesis and the results from Chapter Two, it is worth considering if there are patterns in population mixing between individuals infected with SfMNPV and those that are not. Like other nucleopolyhedroviruses, SfMNPV can be passed vertically between generations through reproduction, such that larvae with SfMNPV are most likely to have obtained their infection from their parents (Vilaplana et al., 2008; Vilaplana et al., 2010).

Therefore, population mixing across Africa could be important in spreading SfMNPV, which is present as a natural enemy in Africa (A. Withers et al., *in review*).

To improve current understanding of population movements of FAW in Africa, in this study we explored population genetic structure across FAW larvae collected from six different African countries (Malawi, Rwanda, Kenya, Sudan, Kenya and Ghana) between 2017 and 2019. We used traditional strain and haplotyping methods for FAW (COIB and TPI), as well as eight highly variable FAW microsatellite loci to determine genetic structure and mixing across countries. Furthermore, we applied a novel approach to studying disease prevalence by using microsatellite markers to determine whether FAW population mixing in Africa reflects SfMNPV movements to improve our understanding of the distribution of natural enemies in FAW in Africa.

3.2.1 Hypotheses

This chapter tests the following hypotheses about FAW in Africa:

1. Microsatellites will be able to infer finer genetic differentiation than the Tpi and COIB markers.
2. FAW will be undergoing long-distance flights across the continent like they do in their native region of North America, and like similar species, such as *Spodoptera exempta,* do in Africa.
3. FAW infected with SfMNPV cluster together more closely than those not infected with SfMNPV due to transmission within populations and vertical transmission.

3.3 Methods

3.3.1 Sample collection

FAW larvae for microsatellite analysis were collected from Ghana, Kenya, Malawi, Rwanda, Sudan, and Zambia. Collection details are provided in Table 3.1 for samples used in microsatellite analysis. FAW larvae for sequencing with COIB:TpiI4:TpiE4 were collected from Ghana (N = 72:70:72, 2017), Malawi (N = 95:27:40, 2018 and 2019), Rwanda (N = 127:127:141, 2017), Sudan (28:28:24, 2017) and Zambia (N = 44:53:34, 2017) and stored in ethanol. Full collection details are provided in Table 3.4.

Table 3.1 FAW larvae collection details. All larvae were collected at instar 3 or 4 and stored in ethanol at -20°C. If the coordinates were not recorded, then it was estimated using a central point for the nearest named town or province. These are the same samples as used in Chapter 2. Microsatellites were amplified from 16 samples in each country. N shows the number of samples included in the study.

Country	Location	Collector*	N	Crop	Date	Longitude	Latitude
Ghana	UCC	A	8	Maize	16/10/2017	-0.16	5.68
Ghana	Twifo Ayaase	A	8	Maize	14/08/2017	-1.49	5.47
Kenya	Embu	B	7	Maize	19/06/2019	34.58	-0.43
Kenya	Homa Bay	B	6	Maize	19/06/2019	37.60	-0.49
Malawi	Salima	C	8	Maize	28/01/2019	34.15	-13.4
Malawi	Thyolo	C	8	Maize	17/09/2018	35.07	-15.92
Rwanda	Kayonza	D	8	Maize	03/05/2017	30.51	-1.91
Rwanda	Nygatare	D	8	Maize	05/05/2017	30.33	-1.29
Sudan	Al Qadarif	F	15	Sorghum	01/09/2017	35.38	14.04
Zambia	Northern Province	E	8	Maize	15/05/2017	31.19	-10.65
Zambia	Lusaka (near Lufansa)	E	8	Maize	20/01/2017	28.32	-15.4

*A: Ben Mensah, B: Aislinn Pearson, Kentosse Ouma and Sevgan Subramanian, C: Donald Kachigamba and Amy Withers, D: Patrick Karangwa, and Bellancille Uzayisenga, E: Gilson Chipabika, Miyanda Moonga, Phillip Nkunika and Kenneth Wilson F: Guillaume Sneessens

3.3.2 DNA extraction

DNA was extracted from samples following the standard protocol for tissue in the Qiagen DNeasy Blood and Tissue kit. DNA was stored in buffer AE at -20°C.

3.3.3 Covert SfMNPV detection

Samples were screened for the presence of covert SfMNPV using standard PCR. The primer sequences and cycling parameters for each infection tested are shown in Table 3.2. Each PCR reaction was carried out in a 0.2ml PCR tube (Fisherbrand) containing 1µl EasyTaq® Buffer (Transgen biotech), 0.5µl 10µM forward primer, 0.5µl 10µM reverse primer, 0.2µl 10mM dNTPs (Transgen biotech), 0.05µl EasyTaq® DNA Polymerase (Transgen biotech), 5.75µl H_2O

and 1µl DNA. Each PCR was carried out twice as the pathogen DNA may be at very low concentrations.

3.3.4 Strain identification and haplotyping using COIB and TPI markers

DNA was amplified for strain identification using COIB and TPI markers for FAW larvae samples (Table 3.2). Following COIB amplification, the product was incubated at 37°C for two hours with 1µl EcoRV restriction enzyme and 2µl NEBuffer to determine FAW strain. EcoRV cuts the amplicon at position 1182bp if it is from the rice strain resulting in two visible bands, and does not cut if it is the corn strain resulting in one larger band when the product is run on a gel electrophoresis. To visualise this, electrophoresis was used with samples loaded into a 1% agarose gel at 125 volts for 45 minutes following the restriction enzyme digest. There are five known haplotypes of the COIB marker, these are corn h1 ($A_{1164}A_{1287}$), corn h2 ($A_{1164}G_{1287}$), corn h3 ($G_{1164}A_{1287}$), corn h4 ($G_{1164}G_{1287}$) and rice ($T_{1164}A_{1287}$) (Nagoshi et al., 2017b) and unidirectional sequencing was used to determine which haplotypes the COIB corn strain samples were. Sequencing reactions contained 0.75 µL BigDye® Reaction Mix, 1.70 µL 5x BigDye® Sequencing Buffer, 0.32 µL 10 µM Forward primer, 5-20 ng template DNA and H_2O to supplement the reaction to 10 µL. The sequencing reaction was preincubated for 1 min at 96° C followed by 25 cycles of: 10 seconds at 96° C; 5 seconds at 50° C; 4 minutes at 60° C. Excess incorporated dye-terminators were removed using EDTA/Ethanol precipitation before resuspending in 13 µL Hi-Di® formamide and capillary gel electrophoresis on an ABI 3500 Genetic Analyzer. Strain identification was carried out using the Tpi marker by Sanger sequencing following the same protocol as for COIB and is based on nucleotide variation in exon-4 (TpiE4), where the corn strain has C_{183}, the R strain has a T_{183} and hybrids (males only) have C/T_{183} (Nagoshi et al., 2017b). Tpi Intron 4 (TpiI4) was used to determine Tpi14 haplotypes based on 18 previously recorded highly variable positions (Nagoshi and Meagher, 2016; Nagoshi et al., 2019a). For sequencing analysis, raw sequences were assembled and aligned using ClustalW in BioEdit (Thompson et al., 1994; Hall, 1999). Statistical analysis on strain and haplotype distributions were carried out in R using a Poisson GLM followed by a Chi^2 test using the ANOVA function. Based on the TpiI4 haplotypes an AMOVA was carried out using the POPPR package as this gave details of the variance explained within and between samples and populations (Kamvar et al., 2014), from which a P value was calculated using a randomization test with 999 permutations.

Table 3.2 Primer information for COIB and TPI strain identification and haplotyping, and the detection of covert SfMNPV. DNA was extracted from FAW larvae collected in Africa and these primers were used for population analysis (COIB and TPI) and for the detection of covert SfMNPV in this study.

Primer	Expected product size (bp)	F primer	R primer	Cycling parameters
Spodoptera frugiperda nucleopolyhedrovirus: Sfpg41.1 gene (Simón et al., 2008)	650 – 750 (some variation between genotypes)	5'-CGACAATGTCATCGTCTTCG	5'-ATATGTTAGTGGTGGCGGAC	95°C 2min, 30 cycles of 95°C 30sec, 52°C 30sec, 68°C 45sec and then a final extension of 68°C for 5min
COIB (891 – 1472) (Nagoshi et al., 2017b)	603bp	5'-TACACGAGCATATTTTACATC	5'-GCTGGTGGTAAATTTTGATATC	94°C 10min, 33 cycles of 94°C 1min, 55°C 1min, 72°C 1min and then a final extension of 72°C for 5min
TPI designed by de Boer (published in Withers *et al.* 2021) and based on (Nagoshi and Meagher, 2016)	814bp	5' – ATGATTAGGACATCGGAGC	5'-ATGTAATCCAGTCAATGCCTA	94°C 10min, 33 cycles of 94°C 1min, 55°C 1min, 72°C 1min and then a final extension of 72°C for 5min

3.3.5 Microsatellite amplification

Eight highly-variable microsatellites were selected for amplification, as shown in Table 3.3 (Arias et al., 2011; Arias et al., 2019). Each sample was amplified in 20µl reactions composed of 2µl EasyTaq® Buffer (Transgen biotech), 1µl 10µM forward primer, 1µl 10µM reverse primer, 0.4µl 10mM dNTPs (Transgen biotech), 0.1µl EasyTaq® DNA Polymerase (Transgen biotech), 13.5µl H_2O and 2µl DNA. Amplification conditions were 95°C for 1 minute, 30 cycles of 95°C 30 seconds, 60°C 30 seconds, 68°C seconds and a final extension of 68°C for 5 minutes. Once amplified, samples were stored at 20°C until ready for genotyping.

Table 3.3 Microsatellite primer details. The following primers were selected for use in this study to determine population structure in FAW larvae collected in Africa (Arias et al. 2011 and 2019).

Name	GenBank identification	Simple sequence repeat (SSR)	Forward primer (5'-3')	Reverse primer (5'-3')
Spf343	HM752609	(TG)12	[6FAM]GTCAAAGTTTTACATGGAAGCGTG	CCCATCTGTTTGTCCACAGTAAAG
Spf670	HM752637	(CAT)5	[6FAM]GGGAGAGGTTTCTAGCTTCTACGG	GAGGAGCCTTGGTTCAATAGTGC
Spf789	HM752653	(CACAC)4	[6FAM]CGACACGTTGATTGCTCACAG	AATCTTTTATCACAATTCGCAGCC
Spf918	HM752666	(TG)6	[6FAM]GCGAAATTGTTTTAATGTGGGTTG	ACGACCTATACGGACCTTGTTACG
Spf997	HM752675	(TACA)4	[6FAM]TTGATGCATGAATTTTCAAACGAG	ATCACGTTGTGGTCCAATCAATG
Spf1502	HM752731	(CA)12	[6FAM]TTTGCAATTTTAGTTACAAACGTCCTC	TATTGATAGCCTCGTGTTTGACCC
Spf1592	HM752740	(TG)10	[6FAM]GGTTCCTGTTATCACCTGCAGTA	CTATGTAGTTTATGTTAATTCGCACGAT
Spf1706	HM752751	(AC)9	[6FAM]CCACTGTACTGTGATAAACAGATGGC	ATGATCATACAAAGTGCATCCGTG

3.3.6 Microsatellite genotyping

Fragment genotyping was carried out on an ABI3500 sequencer. Each reaction was composed of 11µl HiDi Formamide, 0.4µl Rox500 size standard and 1µl PCR product (Spf343, Spf997 and Spf1706) or 0.5µl PCR product (Spf1592, Spf670, Spf789, Spf918, Spf1502). Genotyping results were viewed on Thermo Fisher Connect™. The cut off for successful amplification was >100RFU, and for heterozygotes the minor peak was >50% of the major peak. An allele was categorised as a specific marker size if the RFU was within a 0.5 range (for example, a size of 150.2 and 150.6 were both classed as 150).

3.3.7 Microsatellite analysis

Samples with fewer than 5 microsatellites amplified were removed from the analysis. Microsatellite analysis was carried out in R (v. 4.0.3) (R Core Team, 2020). Hardy-Weinberg equilibrium was tested using the PEGAS R package (Paradis, 2010). The frequency of null alleles was determined using the Chakraborty et al (1994) formula through the POPGENREPORT R package (Adamack and Gruber, 2014). Heterozygosity and F-statistics were calculated using the HIERFSTAT package (GOUDET, 2005). Genetic differentiation was measured using the MMOD package (G_{st} and Jost's D) (Winter, 2012). Linkage disequilibrium was calculated using an association index using the POPPR package (Kamvar et al., 2014) and by composite linkage disequilibrium using GenePop (v 4.7) (Raymond and Rousset, 1995). An Analysis of Molecular Variance (AMOVA) to determine population differentiation based on genetic distance was carried out using the adonis2 function from the VEGAN package in R for all variables (Oksanen et al., 2012) and country was looked at using an AMOVA with the POPPR package as this gave details of the variance explained within and between samples and populations (Kamvar et al., 2014). To identify population clusters, a Discriminant Analysis of Principle Components (DAPC) was carried out after clusters were identified de novo (i.e., no prior location information) using the *find.clusters* function in the ADEGENET package in R (Jombart, 2008). Optimum number of K was selected based on BIC (Fig. 3.5). The number of PCs retained in the DAPC for both 3 and 4 cluster analysis was 7, this was determined using the a-score with the *optim.a.score* function in the ADEGENET package in R (Fig. 3.1). STRUCTURE (v. 2.3.4) was used to identify population clusters, using an admixture model with 100,000 burnin and 100,000 reps for K1 to K15 with 15 iterations per K (Pritchard et al., 2000). STRUCTURE results were visualised using STRUCTURE HARVESTER (Earl and vonHoldt, 2012), CLUMPP (Jakobsson and Rosenberg, 2007) and DISTRUCT (Rosenberg, 2004).

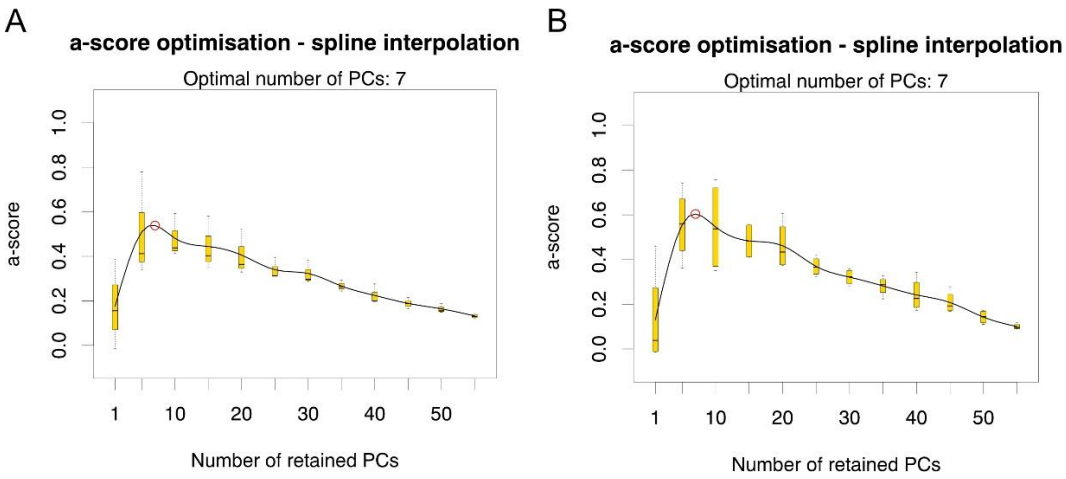


Figure 3.1 The results of the a-score optimisation test to determine the number of principal components to retain in the DAPC analysis. A) For DAPC analysis with 3 clusters and B) for DAPC analysis with 4 clusters.

3.4 Results

3.4.1 Strain identification and haplotyping using COIB and TpiE4 markers

The expected strain discordance between the COIB and TpiE4 markers was observed in all countries, with the markers only reporting the same strain in 19% of samples (Table 3.4). In all countries both markers identified both corn and rice strain FAW (Fig. 3.2). Overall, the COIB strain most frequently reported samples as the rice strain (mean ±S.E. = 72%±0.09), whereas the TpiE4 marker reported them as the corn strain (mean ±S.E. = 92%±0.02). Both markers showed very similar strain frequencies across Malawi, Rwanda, Sudan and Zambia. In Ghana more samples were reported as corn strain (63%) than rice using the COIB marker compared to the other countries, and country had a significant effect on the distribution of the corn strain based on the COIB marker (χ_4=53.17, *P*<0.001). However, when using the TpiE4 marker the proportion reported as corn was very similar across all five countries (χ_4=1.16, *P*=0.885). Those larvae identified as corn by the COIB marker in Ghana (N=45), Rwanda (N=16), Sudan (N=6) and Zambia (N=8) were sequenced to determine haplotype, all larvae were identified as corn CSh4 suggesting very little genetic differentiation based on COIB haplotypes.

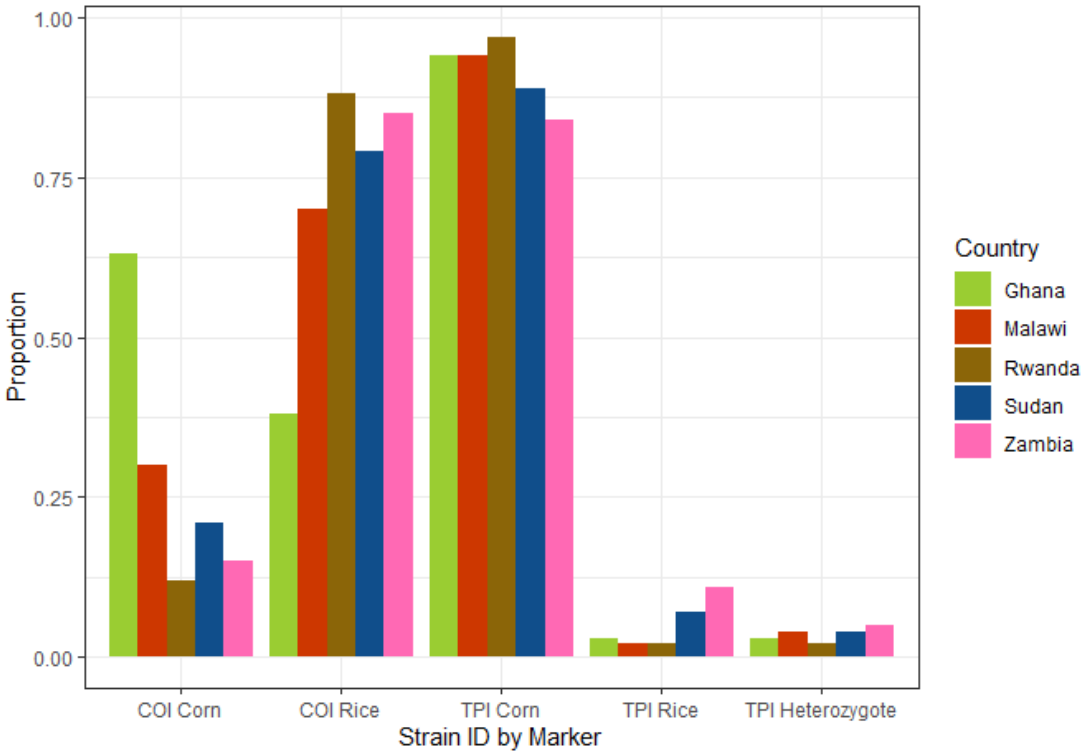


Figure 3.2 Strain identification of FAW larvae using COIB and TPIE4 markers. The COIB marker identified most samples as rice strain, whereas the TPI marker identified most of the same samples as corn strain. The number of samples for COIB:TPIE4 markers tested per country are Ghana 72:72, Malawi 40:95, Rwanda 127:126, Sudan 28:28 and Zambia 53:44.

Table 3.4 Strain identification using COIB and TpiE4 markers. Fall armyworm larvae were collected throughout Africa and collection details are show in the table below. DNA was extracted from larvae and genetic markers were used to determine if the larvae were corn or rice strain. The Tpi and COIB markers showed disagreement in strain identification, and the percentage of samples the markers showed the same result for is shown in the table.

Country	Location	Year	Number	TpiE4 Marker				Tpil4 Marker			COIB Marker								Markers (Tpil4 and COIB) Agree (%)
				Number	corn	rice	het	Number	corn	rice	Number	corn	rice	Number haplotyped	H1	H2	H3	H4	
Ghana	Twifo Ayaase	2017	30	30	0.93	0.03	0.03	29	0.93	0.07	30	0.83	0.17	25	0	0	0	25	0
Ghana	Aviation Farm	2017	4	4	1.00	0.00	0.00	4	1.00	0.00	4	0.75	0.25	3	0	0	0	3	3
Ghana	University Cape Coast	2017	38	38	0.95	0.03	0.03	37	0.97	0.03	38	0.45	0.55	17	0	0	0	17	16
Ghana	All	2017	72	72	0.94	0.03	0.03	70	0.96	0.04	72	0.63	0.38	45	0	0	0	45	19
Malawi	Thyolo	2018	20	20	0.95	0.05	0.00	20	0.95	0.05	20	0.35	0.65	0	0	0	0	0	8
Malawi	Nchalo	2018	20	16	1.00	0.00	0.00	7	1.00	0.00	20	0.25	0.75	0	0	0	0	0	2
Malawi	Salima	2019	30	29	0.90	0.00	0.10	NA	NA	NA	0	NA	NA	NA	NA	NA	NA	NA	NA
Malawi	Lilongwe	2019	31	30	0.93	0.03	0.03	NA	NA	NA	0	NA	NA	NA	NA	NA	NA	NA	NA
Malawi	All	2018/2019	101	95	0.94	0.02	0.04	27	0.96	0.04	40	0.30	0.70	0	0	0	0	0	10
Rwanda	Ruhango	2017	9	9	1.00	0.00	0.00	8	1.00	0.00	9	0.00	1.00	0	NA	NA	NA	NA	0
Rwanda	Nyanza	2017	10	10	0.80	0.20	0.00	9	0.89	0.11	10	0.10	0.90	2	0	0	0	2	3
Rwanda	Gisagara	2017	10	10	1.00	0.00	0.00	10	1.00	0.00	10	0.20	0.80	2	0	0	0	2	2
Rwanda	Muhanga	2017	8	8	1.00	0.00	0.00	8	1.00	0.00	8	0.13	0.88	1	0	0	0	1	1
Rwanda	Kirehe	2017	10	10	1.00	0.00	0.00	8	1.00	0.00	10	0.30	0.70	3	0	0	0	3	3
Rwanda	Gatsibo	2017	10	10	1.00	0.00	0.00	7	1.00	0.00	10	0.10	0.90	1	0	0	0	1	1
Rwanda	Kayonza	2017	10	10	0.90	0.00	0.10	8	1.00	0.00	10	0.10	0.90	1	0	0	0	1	1
Rwanda	Nyagatare	2017	10	10	0.90	0.00	0.10	10	0.90	0.10	10	0.20	0.80	2	0	0	0	2	2

Rwanda	Rusizi	2017	10	10	1.00	0.00	0.00	10	1.00	0.00	10	0.30	0.70	3	0	0	0	3	3
Rwanda	Nyamasheke	2017	10	10	1.00	0.00	0.00	10	1.00	0.00	10	0.10	0.90	1	0	0	0	1	1
Rwanda	Karongi	2017	10	9	1.00	0.00	0.00	9	1.00	0.00	10	0.00	1.00	0	0	0	0	0	0
Rwanda	Ngororero	2017	10	10	1.00	0.00	0.00	10	1.00	0.00	10	0.00	1.00	0	0	0	0	0	0
Rwanda	Kamonyi	2017	10	10	1.00	0.00	0.00	10	1.00	0.00	10	0.00	1.00	0	0	0	0	0	0
Rwanda	All	2017	127	126	0.97	0.02	0.02	141	0.99	0.01	127	0.12	0.88	16	0	0	0	16	17
Sudan	Al Qadarif	2017	28	28	0.89	0.07	0.04	24	0.96	0.04	28	0.21	0.79	6	0	0	0	6	7
Zambia	Lusaka	2017	15	15	0.73	0.20	0.07	13	0.69	0.31	15	0.47	0.53	7	0	0	0	7	6
Zambia	Western Province	2017	3	1	1.00	0.00	0.00	NA	NA	NA	3	0.00	1.00	0	NA	NA	NA	NA	0
Zambia	Eastern Province	2017	4	4	0.50	0.50	0.00	4	0.50	0.50	4	0.00	1.00	0	NA	NA	NA	NA	2
Zambia	Southern Province	2017	3	3	0.67	0.00	0.33	2	1.00	0.00	3	0.33	0.67	1	0	0	0	1	1
Zambia	Northern Province	2017	10	10	1.00	0.00	0.00	9	1.00	0.00	10	0.00	1.00	0	NA	NA	NA	NA	0
Zambia	North-West Province	2017	8	4	1.00	0.00	0.00	NA	NA	NA	8	0.00	1.00	0	NA	NA	NA	NA	0
Zambia	Luapula	2017	10	7	1.00	0.00	0.00	6	1.00	0.00	10	0.00	1.00	0	NA	NA	NA	NA	0
Zambia	All	2017	53	44	0.84	0.11	0.05	34	0.82	0.18	53	0.15	0.85	8	0	0	0	8	9

3.4.2 Strain haplotyping using TpiI4 markers

In all countries this marker identified both corn and rice strain FAW, with the corn strain (82 to 99%) more common compared to the rice strain (1% - 18%) (Fig. 3.3). The most common haplotype in every country haplotype was TpiCa1a, and the rarest was TpiCa2C (Fig. 3.3). A novel rice haplotype was identified in samples from Malawi, Rwanda and Sudan, where no larvae were of the previously recorded rice haplotype (Fig. 3.3). The greatest number of different haplotypes was observed in Ghana, with four different haplotypes identified. Heterozygotes were recorded in all countries, however due to ambiguity in which haplotype combinations these were they were only identified as heterozygotes (Fig. 3.3). An AMOVA was carried out on the TpiI4 alignment and showed significant differences between the six countries, however the total variance explained by differences between countries was low, with most of the genetic variation being between individuals within countries, which would suggest a largely panmictic population (Table 3.5).

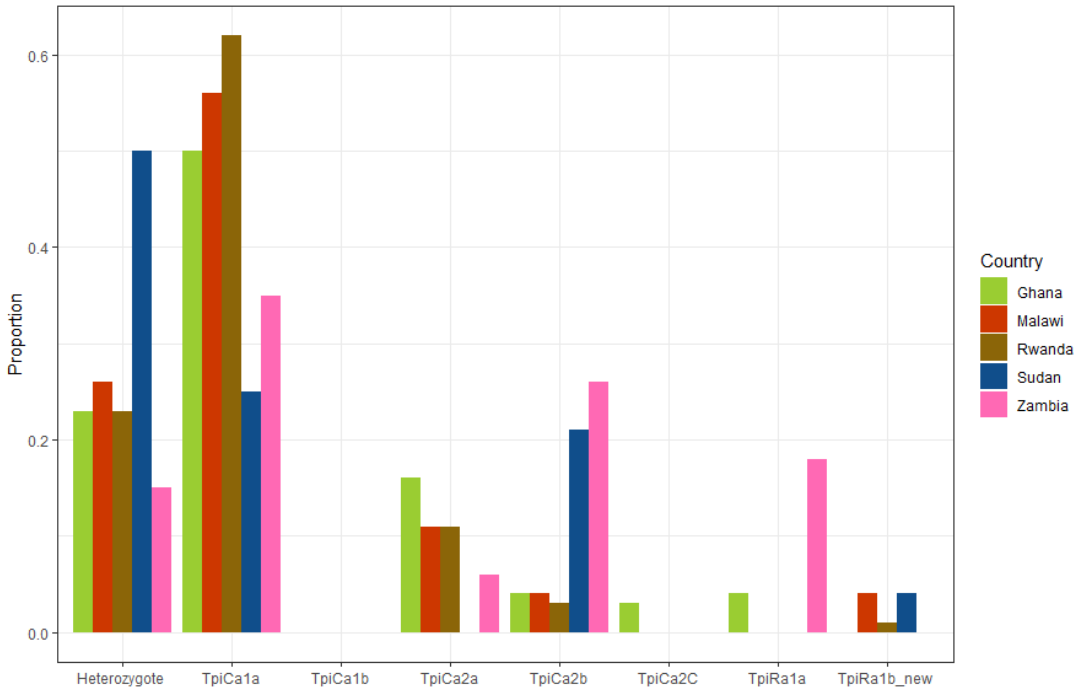


Figure 3.3 Haplotype identification of FAW larvae using TpiI4 markers. The TpiI4 marker identified most larvae as the corn strain, with the most common haplotype being TpiCa1a. Heterozygotes of the rice and corn haplotype were detected in all countries, and due to ambiguity the parental haplotypes could not be determine for these larvae. The number of samples for each country are Ghana 70, Malawi 27, Rwanda 141, Sudan 24 and Zambia 34.

Table 3.5 Results of an AMOVA to analyse differences between the six countries in this analysis based on TpiI4. Negative variance components occur in an AMOVA when there is no evidence of genetic structure. *P* value was calculated using a randomization test with 999 permutations.

Variation	**Df**	**Sum of Squares**	**Variance components**	**Total variance (%)**	***P* value**
Between countries	4	13.40	0.04	3.51	0.002
Between individuals within countries	292	342.66	1.17	96.49	NA
Total	296	356.06	1.22	100	NA

3.4.3 Locus information

All eight microsatellites successfully amplified, and the number of alleles found ranged from 3 to 13 (Table 3.6). Twenty-one individuals (23%) had missing allele data; this ranged from 1 to 3 loci per individual, with an average of 0.34 (Table 3.6). Null allele frequencies were high for four loci; Spf1502, Spf343, Spf997 and Spf670 (Table 3.6). Seven of the eight microsatellites significantly deviated from *Hardy-Weinberg equilibrium* (HWE) when all individuals were considered together (Table 3.6). However, some of these microsatellites were in HWE at the within-country level (Table 3.7). The *index of association* (rbarD) metric measures how likely individuals are to be the same at one particular loci in relation to other loci, and how this compares to other individuals, and can give a good indication of linkage between loci (Agapow and Burt, 2001). This metric was calculated and suggested a possible high chance of linkage between three pairs of loci (Fig. 3.4). However, a composite linkage disequilibrium test, which measures the association between two alleles (Weir, 1996), did not find any significant evidence of linkage disequilibrium (Table 3.8).

Table 3.6 Locus and allele information for each of the eight microsatellites. Those loci with high null allele frequencies are in italics. Loci which significantly deviate from HWE are in bold italics and were calculated using a Monte Carlo Exact Test.

Locus	Individuals	Number of alleles	Number of individuals with locus missing data	Allele size range (bp)	Null allele frequency	Hardy-Weinberg Equilibrium *P*
Spf1502	*82*	*10*	*10*	*124 - 141*	*0.62*	***<0.001***
Spf789	86	13	5	182 - 199	0.11	**<0.001**
Spf343	*91*	*8*	*1*	*107 - 127*	*0.35*	***<0.001***
Spf997	*90*	*7*	*2*	*79 - 113*	*0.23*	***<0.001***
Spf1706	91	3	1	118 - 126	0.16	**<0.001**
Spf1592	87	11	5	187 - 217	0.00	**0.048**
Spf918	88	6	4	111 - 123	0.00	0.578
Spf670	*90*	*7*	*2*	*128 - 152*	*0.45*	***<0.001***

Table 3.7 Hardy-Weinberg equilibrium (HWE) for each locus separated by country. Locus which significantly deviate from HWE are in bold italics, *P* value was calculated using a Monte Carlo Exact Test.

Locus	Ghana	Kenya	Malawi	Rwanda	Sudan	Zambia
Spf1502	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***0.043***	***<0.001***
Spf789	0.114	0.31	***0.049***	0.47	0.958	0.08
Spf343	***<0.001***	0.155	***0.004***	***0.002***	***0.004***	0.01
Spf997	***<0.001***	0.27	0.077	0.611	0.089	***<0.001***
Spf1706	1	1	0.32	0.071	**0.033**	1
Spf1592	0.705	0.716	0.264	0.122	0.727	***0.031***
Spf918	0.332	0.645	0.427	0.603	0.746	0.129
Spf670	***<0.001***	***0.001***	***0.002***	***<0.001***	***<0.001***	***<0.001***

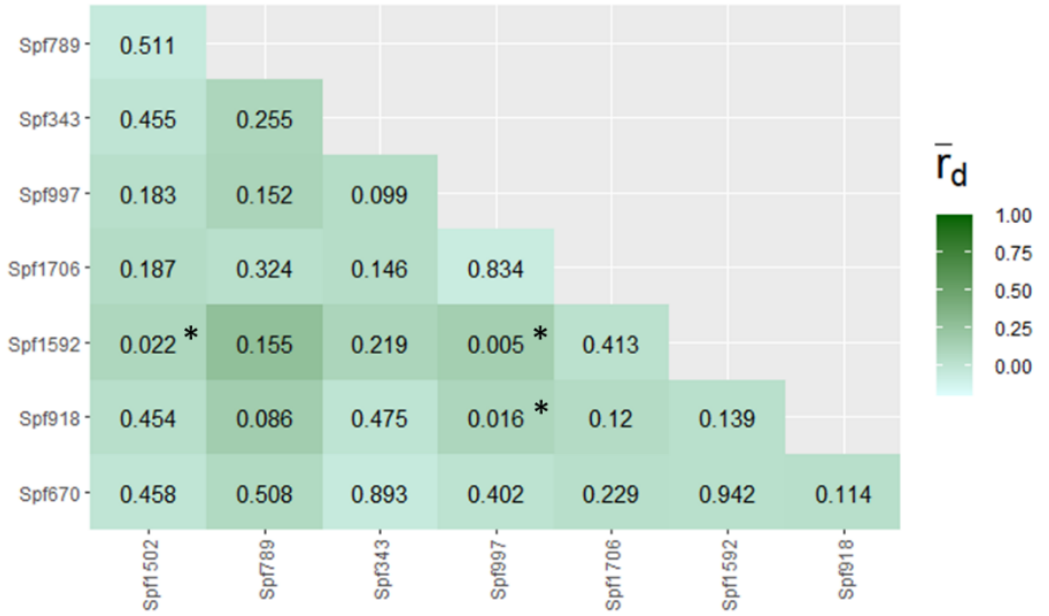


Figure 3.4 Standardised index of association (rbarD) for each pair of loci, with darker green representing higher index of associations. Numbers are *P* values, and those with asterisks are significant at *P*<0.05.

Table 3.8 Composite linkage disequilibrium *P* value for each pair of loci. No pairs showed significant evidence of linkage between alleles.

	Spf1502	**Spf789**	**Spf343**	**Spf997**	**Spf1706**	**Spf1592**	**Spf918**	**Spf670**
Spf1502								
Spf789	0.921							
Spf343	0.998	0.942						
Spf997	0.196	0.797	0.547					
Spf1706	0.568	0.700	0.828	0.968				
Spf1592	0.982	1.000	0.076	0.999	0.568			
Spf918	0.707	0.268	0.058	0.726	0.565	0.414		
Spf670	0.088	0.849	0.065	0.403	0.949	0.778	0.788	

3.4.4 Population differentiation

Population differentiation can be measured in several ways; here we used three common measures (Nei's *Gst*, Hedrick's *Gst* and *Jost's D*) and each suggested that there was very little evidence of population differentiation across Africa (Nei, 1973; Hedrick, 2005; Jost, 2008) (Table 3.9). In all three measures tested, a value of 0 suggests little genetic differentiation (panmixia) and 1 suggests high levels of segregation. The range of the three measures across all loci was 0.03 to 0.14 (Table 3.9). There was also evidence of low genetic variance based on *Fst* between countries at each locus tested (Table 3.9). The level of inbreeding occurring within populations can be inferred from *Fis*, however, this varied between loci with high levels suggested for some loci (e.g. Spf1502 and Spf670), but low for others (e.g. Spf1592 and

Spf789) (Table 3.9). Pairwise *Fst* values between the six countries ranged from -0.02 to 0.08 (mean ± S. E. = 0.03 ± 0.01) suggesting high levels of population mixing (Table 3.10). These results suggest that, in Africa, FAW may frequently mix with FAW from other countries suggesting very little population differentiation is occurring.

Table 3.9 Genetic differentiation measures for FAW in Africa based on the eight microsatellites. In all three measures tested, a value of 0 suggests very little genetic differentiation (panmixia) and 1 suggests high levels of segregation. All measures are based on *Hs* (heterozygosity within populations) and *Ht* (heterozygosity without population structure). F-statistics represent genetic variance in a subpopulation compared to the whole (*Fst* – values closer to 1 suggest high levels of differentiation between populations) or in a subpopulation compared to individuals within that subpopulation (*Fis* – values close to 1 suggest high levels of inbreeding in populations). Negative values of *Fst* and *Fis* should be interpreted as 0 and suggest very low differentiation of populations (*Fst*) or very low chance of inbreeding (*Fis*). Confidence intervals of *Fis* based on bootstrapping are provided.

Locus	Heterozygosity		Population differentiation			F-statistics			
	Hs	***Ht***	***Nei's Gst***	***Hedrick's Gst***	***Jost's D***	***Fst***	***Fis***	***CI Fis (-)***	***CI Fis (+)***
Spf1502	0.76	0.82	0.07	0.36	0.30	0.05	0.75	0.19	0.66
Spf789	0.79	0.89	0.11	0.60	0.54	0.10	0.10	0.03	0.52
Spf343	0.74	0.75	0.01	0.05	0.04	0.00	0.52	0.19	0.61
Spf997	0.67	0.69	0.04	0.14	0.10	0.03	0.35	0.13	0.44
Spf1706	0.18	0.18	0.03	0.04	0.01	0.02	0.26	0.07	0.46
Spf1592	0.85	0.86	0.01	0.10	0.09	0.01	-0.01	0.06	0.54
Spf918	0.64	0.65	0.01	0.03	0.02	0.01	-0.02	0.19	0.66
Spf670	0.79	0.80	0.01	0.05	0.04	-0.01	0.63	0.03	0.52
All	NA	NA	0.04	0.14	0.03	0.03	0.33	NA	NA

Table 3.10 Pairwise *Fst* values for the six countries. Pairwise *Fst* values were calculated based on Nei's method (Nei, 1987).

	Ghana	Kenya	Malawi	Rwanda	Sudan
Kenya	-0.023				
Malawi	-0.004	-0.001			
Rwanda	0.001	-0.014	0.008		
Sudan	0.043	0.041	0.055	0.045	
Zambia	0.070	0.052	0.071	0.082	0.079

Population differentiation was further analysed using an AMOVA to determine if the genetic distance between individuals varies by country, sampling year, location within country or with SfMPNV presence, or an interaction between these two variables (Table 3.11). There was no significant difference between samples from locations within countries ($F_{1,74}$=1.17, *P*=0.119), or between sampling years ($F_{1,74}$=0.96, *P*=0.513). The presence of covert SfMNPV did not significantly influence the genetic distance of FAW, suggesting that populations are equally likely to mix whether they have SfMNPV present or not ($F_{1,74}$=1.03, *P*=0.414). There was no significant interaction between country and disease ($F_{1,74}$=1.19, *P*=0.121). The AMOVA suggested that FAW from each country were genetically different to FAW from other countries ($F_{5,74}$=1.82, *P*=0.001).

Table 3.11 Results of an AMOVA to determine if the genetic distance of FAW in Africa is influenced by the country they are from or the presence of SfMNPV. Significant *P* values are shown in bold italics.

Variable	Df	Sum of Squares	R^2	F	*P*
SfMNPV	1	15.05	0.01	1.03	0.414
Country	5	132.59	0.09	1.82	***0.001***
Year	1	14.04	0.01	0.96	0.513
Location	4	68.46	0.05	1.17	0.119
*SfMNPV * Country*	3	52.35	0.04	1.20	0.122
*SfMNPV * Year*	1	14.57	0.01	1.00	0.440
*SfMNPV * Location*	2	26.90	0.02	0.92	0.655
Residual	74	1078.79	0.77		
Total	91	1402.74	1		

As *Country* was the only significant factor influencing FAW population differentiation, a second AMOVA was carried out to determine variance between and within countries. This suggested significant differences between the six countries, however the total variance explained by differences between countries was low, and most of the genetic variance was found within individuals which would suggest a largely panmictic population (Table 3.12).

Table 3.12 Results of an AMOVA to analyse differences between the six countries in this analysis based on the microsatellites. *P* value was calculated using a randomization test with 999 permutations, and significant *P* values are shown in bold italics.

Variation	Df	Sum of Squares	Variance components	Total variance (%)	*P* value
Between countries	5	69.24	0.197	3.25	***0.001***
Between individuals within countries	86	672.08	1.961	32.41	***0.001***
Within individuals	92	358.17	3.893	64.34	***0.001***
Total	183	1099.49	6.051	100	NA

3.4.5 Population clustering using STRUCTURE identified 3 genetic clusters

Clustering was carried out using an admixture model in *STRUCTURE*, with the number of clusters selected using *Delta K* (Evanno method) and *LnPr(K)* methods [24]. Based on *Delta K* there were three genetically distinct clusters in FAW (Fig. 3.5A and 3.5B). Based on *LnPr(K)*, the most likely number of clusters was five (Fig. 3.5C and 3.5D). Both 3 and 5 clusters show that FAW from Sudan and Zambia were more genetically isolated from the four other countries, though some individuals from the other four countries do show similar assignment patterns suggesting population mixing does occur between all countries. Ghana, Kenya, Malawi and Rwanda appear very similar to each other, suggesting high levels of population mixing between these countries (Fig. 3.5C and 3.5D). Based on the similarities between the structuring results for 3 and 5 clusters, and that the *LnPr(K)* begins to plateau after K=3 we propose that population structure of FAW in Africa is best described by three genetic

clusters. To identify potential substructure in the four most similar countries (Ghana, Kenya, Malawi and Rwanda) they were analysed alone in *STRUCTURE*. This identified 3 genetic clusters as the most likely scenario based on both *DeltaK* and *LnPr(K)* and further confirmed high levels of mixing between the countries, with no strong evidence of substructure identified (Fig. 3.5D to 3.5G).

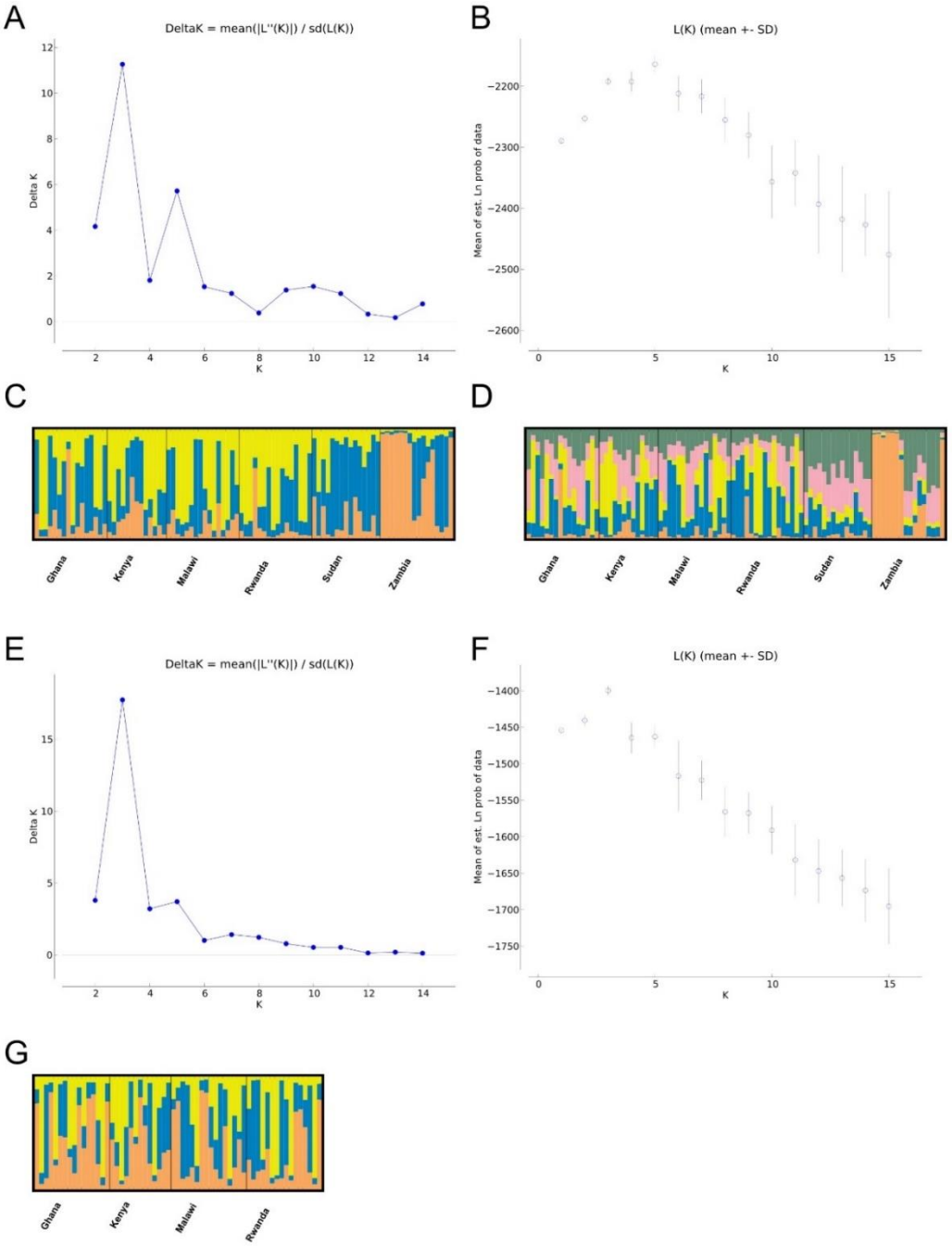


Figure 3.5 Genetic structure of FAW as assigned by *STRUCTURE* analysis of microsatellites. Panels A to D show the results of STRUCTURE with all six countries. Panel A shows the *DeltaK,* Panel B shows the *LnPr(K)* for each cluster, Panel C shows three distinct genetic clusters (the most likely based on *DeltaK*), Panel D shows 5 distinct genetic clusters (the most likely based on *LnPr(K)*). Panels E to G show the results of STRUCTURE carried out to assess substructure hierarchically. Panel E shows the *DeltaK,* Panel F shows the *LnPr(K)* for each cluster and Panel G shows three distinct genetic clusters (the most likely based on both *DeltaK* and *LnPr(K)*).

3.4.6 Population clustering using Discriminant Analysis Principal Components (DAPC) identified 3 genetic clusters

STRUCTURE has been shown to miss some subdivision when clustering individuals (Jombart, 2008), therefore, population clustering analysis was carried out using *Discriminant Analysis Principal Components (DAPC)*. This approach determines the number of possible clusters by running successive K-means clustering, and selecting the most suitable cluster based on Bayesian Information Criterion (Jombart, 2008). This method identified three clusters as the best model based on BIC (BIC=120.67) in FAW when no location information was given prior to running the DAPC, however, four clusters also had a very low BIC (BIC=120.84) which could be considered as a possible alternative (Fig. 3.6).

Based on three clusters, FAW from Sudan were more genetically different to populations from elsewhere in Africa with no individuals assigned to cluster 3, whereas, cluster 3 individuals were found in all other countries (Fig. 3.6). The three clusters highlighted similarities between adjacent countries, Zambia and Malawi, with 50% and 44% of individuals respectively from these countries assigned to cluster 3, and similarities between Kenya and Rwanda, with 23% and 19% of individuals respectively, from cluster 3 identified there (Fig. 3.6). Ghana showed most similarities with Kenya and Rwanda, with 25% of individuals assigned to cluster 3.

SfMNPV was not found in any larvae from Zambia or Kenya whereas covert SfMNPV was detected in 50% of samples from Ghana and Malawi, 20% from Sudan and 13% from Rwanda. The effect of SfMNPV on genetic clustering was investigated based on the three clusters identified using *DAPC* by determining if the individuals assigned to each cluster had covert SfMNPV. This identified no clear clustering based on whether covert SfMNPV was present or absent in the FAW, with individuals harbouring SfMNPV being equally spread over the three clusters (Fig. 3.7).

The *DAPC* was carried out by providing prior information to the model as this can help determine subdivision within populations, so *Country* and *SfMNPV prevalence* (measured as the proportion of samples with SfMNPV) was provided (Fig. 3.8). The clustering of countries was similar to that identified by STRUCTURE, with both Sudan and Zambia genetically different to each other, and to the other four countries. Malawi and Ghana were grouped closely together, as were Rwanda and Kenya (Fig. 3.8). There was some separation of FAW with and without SfMNPV in each country. This provides some evidence that populations with SfMNPV may be genetically isolated from those without (Fig. 3.8).

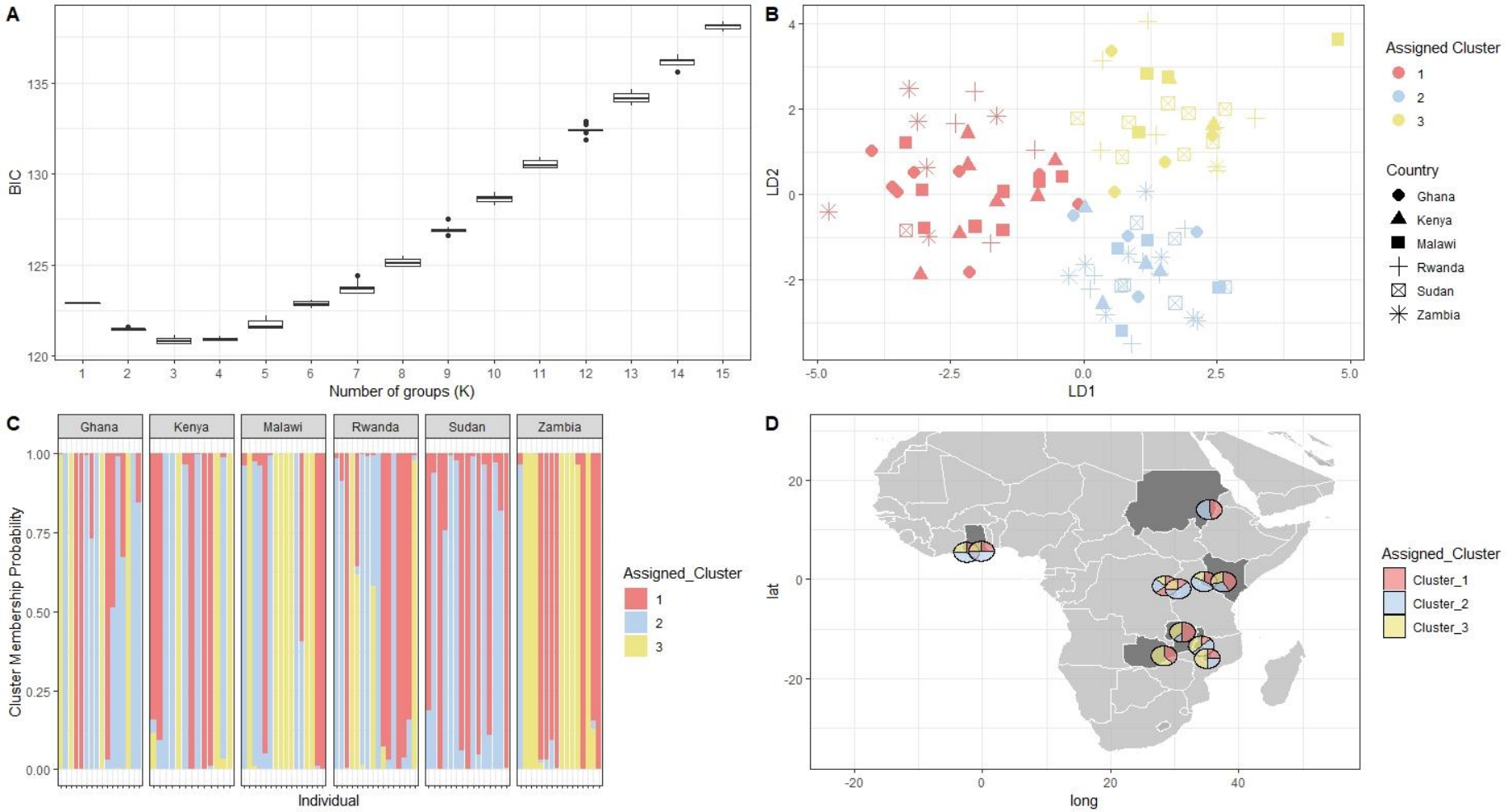


Figure 3.6 *DAPC* clustering (k=3) and assignment of individuals from each country. A) BIC plot for number of groups (K) selection, B) The position of individuals on the first two principal components, and in C) the membership probability of individuals to that cluster. D) Assigned clusters for each sampling location are shown on a map. Combined, this shows a largely panmictic population of FAW across Africa with some possible genetic structuring n between northern and southern populations.

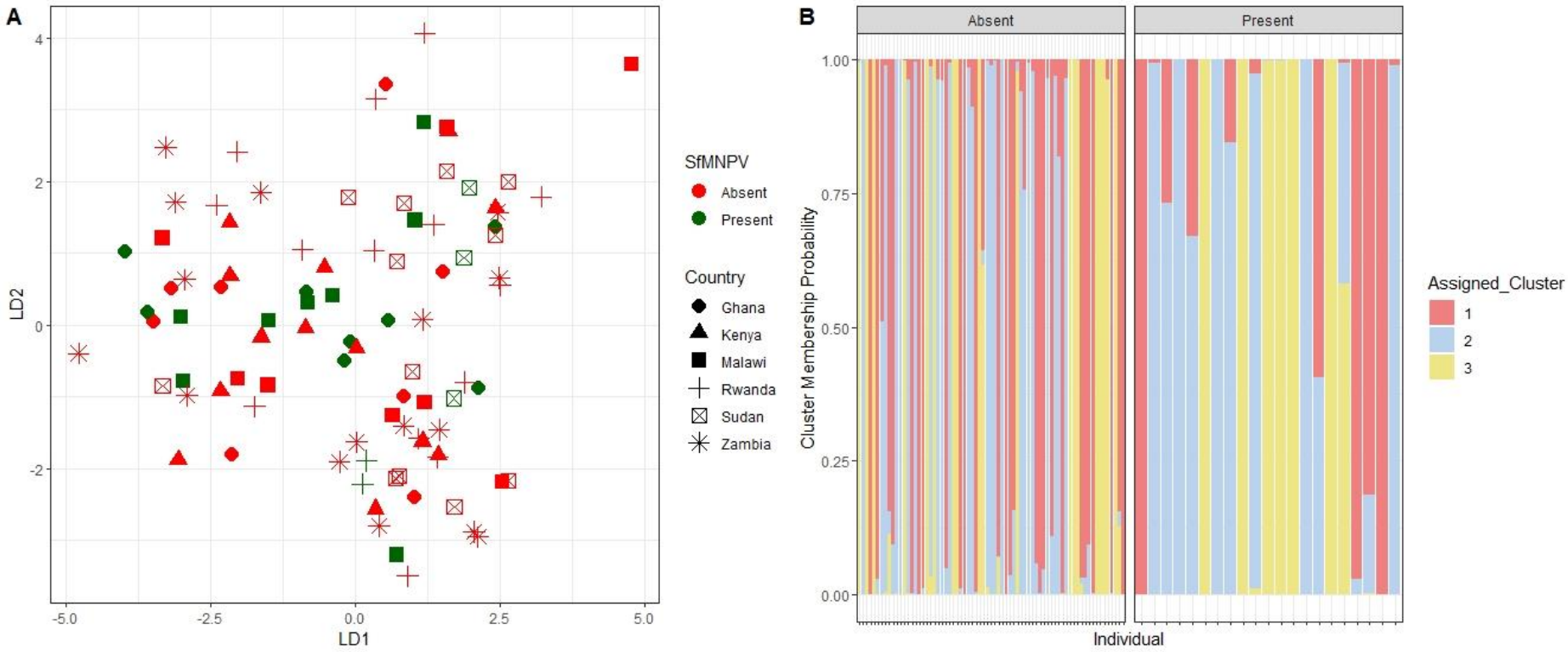


Figure 3.7 *DAPC* clustering (k=3) and assignment of individuals based on covert SfMNPV being present or absent. Shown in A) as position on the first two principal components, and in B) as membership probability of individuals to that cluster. This showed no clear evidence of population clustering based on the presence or absence of SfMNPV.

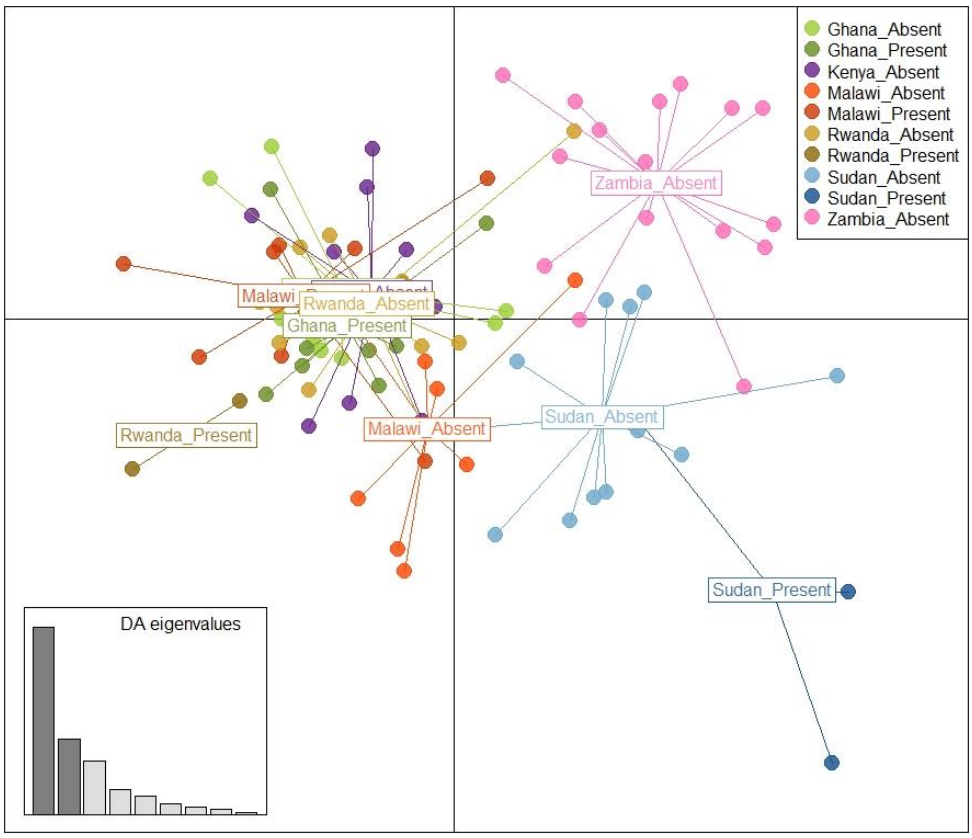


Figure 3.8 *DAPC* clustering and assignment of individuals when location and the presence or absence of SfMNPV was provided to the model, shown as each individual's position on the first two principal components. The eigenvalues for the DAPC are shown inset in the plot, the maximum number was maintained in the analysis and the number of PCs retained was 18 based on the optim a-score of the original model.

3.4.7 Discriminant analysis of principal components (DAPC) using four clusters

When considering four clusters, FAW from Sudan were still more genetically different from those from the other five countries studied, with most individuals assigned to cluster 3 and only one to cluster 1 (Fig. 3.9). Malawi, Ghana and Rwanda were most similar to each other with many individuals from these countries assigned to clusters 3 and 4. Based on the four clusters, Zambia and Kenya were most like each other, suggesting more mixing of FAW populations occurs between these countries than with Sudan, Rwanda and Malawi (Fig. 3.9). However, overall there were fewer clear differences between the countries with more evidence of panmixia than segregation.

The effect of SfMNPV was stronger when considering four clusters, with only two individual harbouring covert SfMNPV being assigned to cluster 2 (i.e., membership probability >50%) suggesting possible genetic differentiation between FAW with and without SfMNPV (Fig. 3.10). This could suggest that FAW with SfMNPV are less likely to mix with other populations that have higher numbers of individuals from cluster 2, such as Zambia and Kenya, both of which had no SfMNPV present.

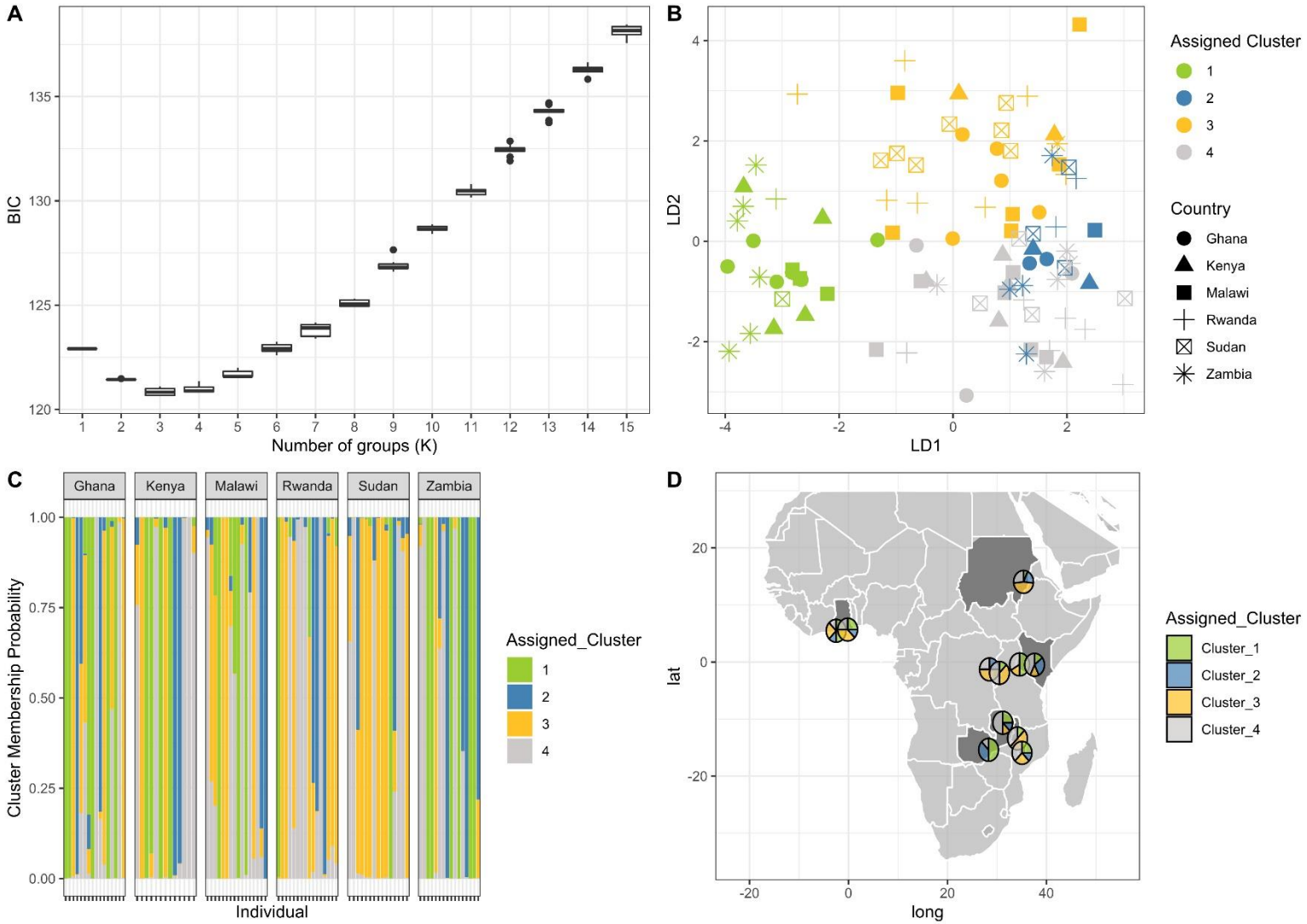


Figure 3.9 *DAPC* clustering (k=4) and assignment of individuals from each country. Shown in A) BIC plot for number of groups (K) selection, B) as position on the first two principal components, and in C) as membership probability of individuals to that cluster. D) Assigned clusters for each sampling location shown on a map. Combined, this shows a largely panmictic population of FAW across Africa with some possible genetic structuring between northern and southern populations.

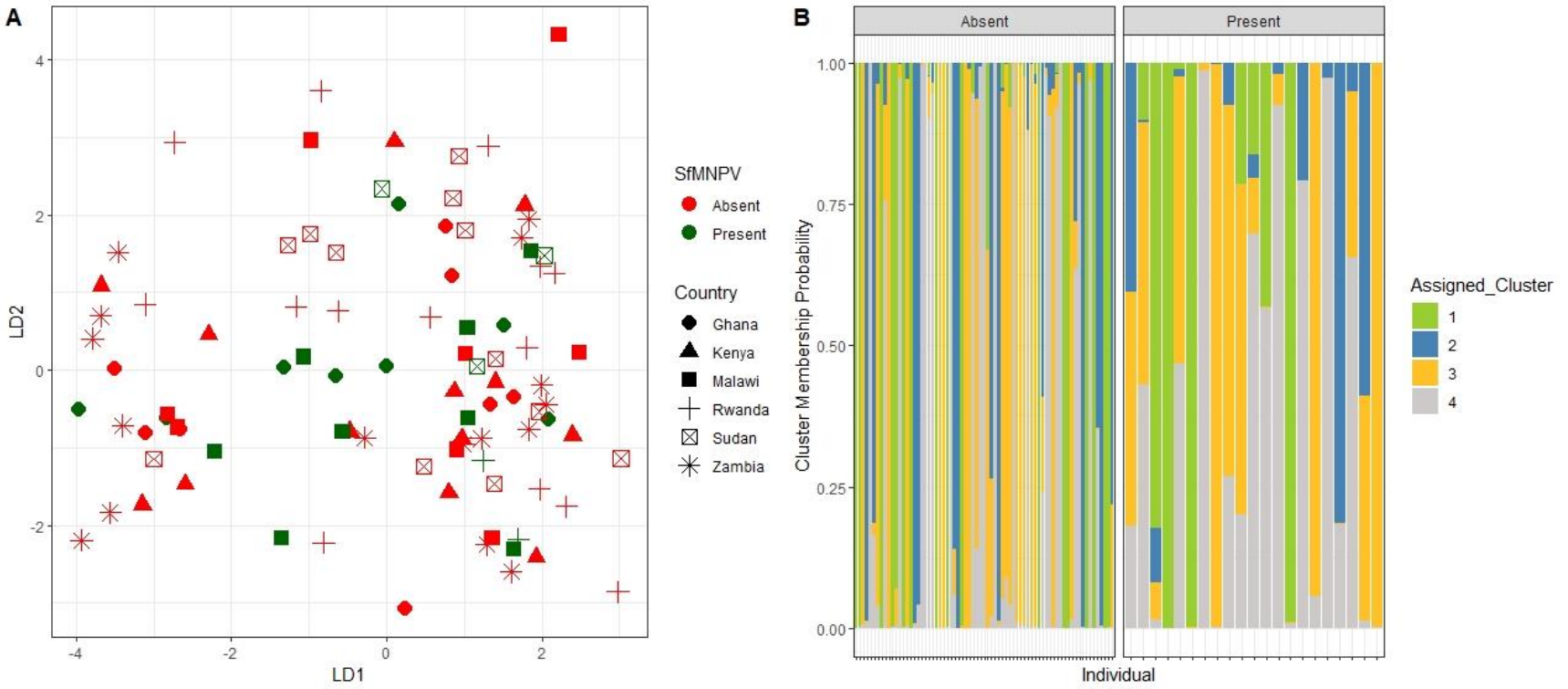


Figure 3.10 *DAPC* clustering (k=4) and assignment of individuals based on covert SfMNPV being present or absent. Shown in A) as position on the first two principal components, and in B) as membership probability of individuals to that cluster. This suggests potential population clustering based on the presence or absence of SfMNPV, with only two individuals with SfMNPV assigned to genetic cluster 2 throughout Africa.

3.5 Discussion

This study is the first to use microsatellites to determine FAW population mixing and genetic diversity in Africa. Considering the unreliability of the COIB and TPI haplotypes for strain identification and the potential for confusion caused by corn and rice strain hybrids (Nagoshi et al., 2017b; Nagoshi et al., 2019a; Nagoshi et al., 2019b; Nagoshi et al., 2020; Zhang et al., 2020; Nayyar et al., 2021), we sought to provide evidence of population structure in FAW in Africa using a microsatellite approach.

The previously reported discordance between the TpiE4 and COIB markers for strain identification was reported in this study, with very little agreement occurring between the markers. Furthermore, based on the COIB haplotypes it was not possible to determine genetic differentiation between the countries as only COIB-h4 was found. The TpiI4 marker showed more variation between the individuals, however, the vast majority of larvae were either TpiCa1a which is in line with previous studies investigating FAW in Africa and Asia (Nagoshi et al., 2019a; Nagoshi et al., 2019b; Nayyar et al., 2021). Previous work based on these markers in Africa concluded that there were significant differences between some African countries with widely separated populations being genetically distinct (Nagoshi et al., 2019b). Whilst the findings here using the TpiI4 marker do support some evidence of genetic variation between countries, this explained very little variance suggesting more of a panmictic population of FAW across Africa.

The low genetic variability observed with the COIB marker, and both TpiE4 and TpiI4 markers, limit the analysis which can be carried out and reduce the likelihood of genetic differentiation between countries being detected. By using highly variable microsatellites, we were able to overcome this challenge to determine genetic differentiation between FAW from different countries in Africa, as well as some similarities suggesting the presence of both resident and migratory populations of FAW throughout the continent.

Most of the microsatellites in this study were out of Hardy-Weinberg equilibrium, whereas in previous population genetics studies using microsatellites for fall armyworm in Paraguay and Brazil, no loci were out of HWE (Arias et al., 2019). However, deviation is expected in invasive populations that have been through a tight bottleneck. The FAW in this study probably originated from a small source population in Africa and will have been through a bottleneck, therefore, this deviation from HWE provides further evidence of a common origin for FAW which then subsequently spread across Africa (Nagoshi et al., 2017b; Nagoshi et al., 2018a). The microsatellites showed evidence of mutation events in the African FAW

compared to populations in Texas, Mississippi, Puerto Rico and Brazil. For example, previously reported allele sizes for locus Spf997 were in the range of 95 to 139 (Arias et al., 2011; Arias et al., 2019), whereas in this study the allele size range for the same locus was 79 to 113. The presence of smaller alleles not previously reported alongside the absence of larger alleles throughout Africa offers more evidence of a genetic bottleneck and loss of genetic diversity. Furthermore, it is likely that if multiple incursion events had occurred then the microsatellites observed here would have matched more closely with those previously recorded.

Though FAW in Africa are likely to have undergone a population bottleneck at the time of invasion, the range of alleles for each locus identified in this study (3 to 13) was similar to that previously reported from Paraguay and Brazil (3 to 15) (Pavinato, 2013; Arias et al., 2019). Based on this range of alleles, previous work found genetic differentiation between northern and southern FAW populations across Brazil and Paraguay, as well as gene flow across all populations sampled (Arias et al., 2019). This indicates that despite a recent bottleneck there is still sufficient genetic diversity in microsatellite regions to enable population genetic studies of FAW in Africa, and supports our hypothesis of microsatellites being able to infer finer genetic differentiation than the Tpi and COI markers.

Populations from the six countries (Kenya, Ghana, Malawi, Rwanda, Sudan and Zambia) did not show strong signs of population differentiation when using traditional measures (Nei's *GST,* Hedrick's *GST* and Jost's *D*). This indicates that the populations mix frequently and no strong genetic structure is present. This was supported by the AMOVA which showed that most of the genetic variance was occurring between individuals.

This lack of population differentiation between countries provides evidence for our hypothesis that FAW are undergoing long distance migratory flights in Africa that create a panmixia of populations. Additionally, there was no evidence of genetic differentiation between samples from different sampling locations within the same country, confirming that populations are mixing within country. This has important consequences for the development of insecticide resistance, as this could mean it would spread rapidly throughout each country and across Africa. This is a very important finding as insecticide resistance (organophosphate and pyrethroid resistance) has already been reported in FAW in China, so is very likely to be present in Africa too (Zhang et al., 2020). Considering the key role that long-distance, migratory flights played in the rapid spread of insecticide resistance both within and across continents in the invasive cotton bollworm (*Helicoverpa armigera*) (Armes

et al., 1996; Buès et al., 2005; Parry et al., 2017; Jones et al., 2019), it is important to consider the implications of frequent, long-distance flights that appear to be occurring in FAW.

The evidence of panmixia contrasts with previous based on the COIB and Tpi markers, which analysed FAW samples from across Africa and found evidence of genetic differentiation between geographically widespread countries. Further investigation with the clustering approaches used in this study show that whilst some countries are similar genetically (e.g. Kenya, Rwanda, and Ghana), others are more differentiated (e.g. Zambia and Sudan). We conclude from this that genetic mixing of FAW populations is occurring across Africa, however, there are some FAW possibly forming resident and partially segregated populations, as seen in parts of South America and the Caribbean (Nagoshi et al., 2017a).

Previous reports suggested a possible east-west divide between FAW populations (Nagoshi et al., 2018a), or no clear pattern of division between populations (Nagoshi et al., 2019b). However, our study using microsatellites found evidence of a north-south divide when samples were assigned to clusters using discriminant analysis. The evidence for this relationship was primarily driven by the number of individuals assigned to certain clusters and their geographical origin (e.g. fewer individuals were assigned to cluster 3 in the north compared to the south). African countries located further south (Zambia, Malawi) showed more similarities to each other than they did to those countries further north (Kenya, Rwanda, Ghana and Sudan). This pattern of genetic separation would tie in well with the known migratory routes of the African armyworm (*S. exempta*) in eastern Africa which follow the movement of the dominant winds each season, typically moving moths towards the north-west or south-west from Kenya, and a more south-westerly movement across southern Africa from Malawi (a figure of African armyworm migration patterns is provided in Appendix C. Fig. S3) (Tucker et al., 1982; Rose et al., 1985). This is aligned with the movement of the inter-tropical convergence zone (ITCZ), with the wind direction (and hence seasonal migration) being broadly south-easterly north of the equator and north-easterly south of the equator (Rose et al., 2000). It is hypothesised that FAW migration may follow the movement of prevailing winds if they were migratory in Africa as, like many other insects, they rely on wind to support high-altitude long-distance flights (Johnson, 1987; Rose et al., 2000; Chapman et al., 2015; Westbrook et al., 2016).

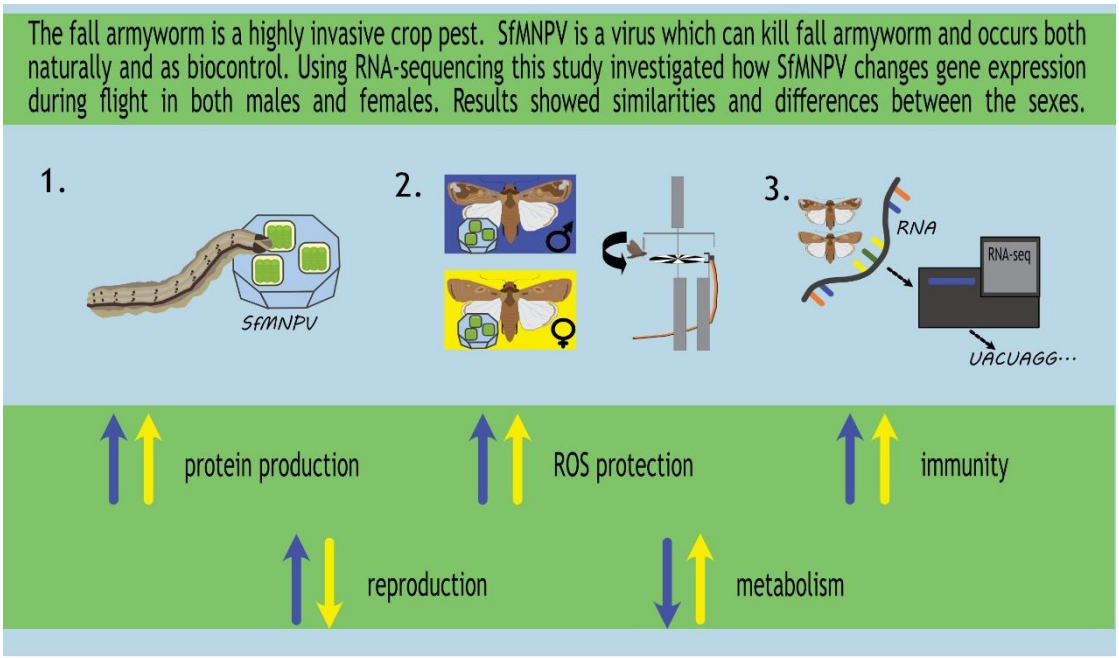
This study considered whether the distribution of a viral natural enemy, SfMNPV, could be linked to population mixing due to the fact that nucleopolyhedroviruses are often

transmitted vertically (Vilaplana et al., 2008; Vilaplana et al., 2010). As we hypothesised, there was some evidence that individuals with SfMNPV were more likely to be grouped with other infected individuals both within and between countries when prior information on SfMNPV infection was provided to the DAPC model. This could suggest that those with SfMNPV may be more likely to mix with other infected FAW. It is hypothesised here that these would be the FAW forming resident populations as infection can reduce flight ability insects and infections can increase more in sedentary populations due to the absence of migratory culling (Chapman et al., 2015; Pearson, 2016). However, in the AMOVA and *DAPC* there was no strong evidence that the prevalence of SfMNPV was linked to populations mixing, which shows that this evidence is currently insufficient to make a strong conclusion and more research is needed.

3.5.1 Conclusion

This study highlights the benefits of using multiple approaches to study genetic diversity, with evidence presented for both widespread genetic mixing between populations alongside some segregation between countries. This is most likely due to a proportion of FAW adults undergoing long-distance migratory flights along the inter-tropical convergence zone whilst the remaining FAW form more sedentary, resident populations. These results provide important evidence that genetic mixing between FAW populations throughout Africa may be more common than previously reported. This has important consequences for FAW management when considering factors such as the spread of insecticide resistance and crop infestations across borders.

Chapter 4 - Gene expression changes related to immunity, reproduction and metabolism during flight are different in male and female fall armyworm.



4.1 Abstract

The fall armyworm (FAW, *Spodoptera frugiperda*) is a highly invasive crop pest, native to North and South America, it has rapidly spread to Africa, Asia and Australasia in recent years. This rapid spread is likely to continue due to its high migratory ability. Biological control using *Spodoptera frugiperda multiple nucleopolyhedrovirus* (*Sf*MNPV) is used to combat fall armyworm outbreaks in the Americas, and has been shown to occur naturally in both the native and introduced range of FAW. Previous work has shown that females are able to maintain strong flight abilities when infected with *Sf*MNPV, whereas the flight ability of males is compromised. To improve our understanding of the interaction between viral infection and migration in fall armyworm, both males and females were challenged with *Sf*MNPV. After five hours of forced flight, RNA was extracted and sequenced to identify transcriptomic changes during flight. There were 159 genes that were differentially expressed in both sexes, of which 106 were upregulated and 53 downregulated. There were more differentially expressed genes in males compared to females; 100 downregulated genes and 257 upregulated genes, compared to 139 downregulated genes and 87 upregulated, respectively. We found that there were similarities and differences between the sexes, for example, protein production and components of the Toll-pathway were upregulated during flight in both sexes. Two immune-related genes with a potential role in the Toll-pathway, a *clip-domain serine protease* and *MYD88*, were significantly differentially expressed between the sexes. Furthermore, metabolic differences were observed between the sexes with genes related to the Krebs' Cycle upregulated only in females and genes involved in lipid metabolism upregulated only in males. Additionally, males appeared to upregulate genes relating to reproduction whereas females downregulated genes relating to reproduction. Overall, this study highlights the transcriptomic variation in the response of each sex to viral challenge and flight, and provides some evidence for the underlying mechanisms driving the behavioural differences in the flight ability of males and females when they are challenged with SfMNPV.

4.2 Introduction

Many migratory insects use flight to travel to new regions. Flight is extremely energy demanding, with wing metabolic rate increasing 50-100 fold (Beenakkers et al., 1984). This energy cost is balanced out because by travelling to different regions individual migrants improve their fitness through moving to more favourable environments (Dingle, 2014). Therefore migration is driven by natural selection and genetics play an important role in migratory behaviour. Understanding this role will improve the current understanding of migratory species and the adaptations that support long distance flight in insects.

Infection is a key driver of natural selection during migration. This is through two processes, 'migratory culling' when individuals are unable to survive migration due to the burden of infection and 'migratory escape' when individuals migrate from areas with higher infection rates to those with lower infection rates (Chapman et al., 2015). These processes have been shown to be important in Lepidoptera, with the migratory monarch butterfly (*Danaus plexippus*) having lower parasite burdens in migrating individuals, reduced parasite prevalence at migration destinations, and shorter, slower flight performances in individuals infected with parasites (Altizer et al., 2000; Bradley and Altizer, 2005).

The impact of infection on flight capacity may vary between the sexes. There was a significant interaction between flight, sex and parasite burden in the monarch butterfly, resulting in male and infected butterflies flying shorter distances than females and uninfected butterflies (Bradley and Altizer, 2005). One explanation for these differences is Bateman's principle, which states that males increase fitness by increasing mating success (i.e., number of matings) whereas females increase fitness by investing more effort into reproduction through longevity (i.e., living longer means more chances to reproduce) (Bateman, 1948). Thus, infected males may fly shorter distances because of investing more into reproduction and mating soon after emergence, whereas females invest more into immunity and flying longer distances as this could increase their fitness through finding favourable habitats for oviposition. Furthermore, there are differences between the sexes at the molecular level following infection and flight. For example, gene expression of immune related genes was higher during flight in the female Glanville fritillary butterfly (*Melitaea cinxia*) compared to males in one population, though the sex differences were not observed in a smaller, isolated population (Kvist et al., 2015). However, it is important to note that the Glanville fritillary is a non-migratory species and so will face different evolutionary pressures. Therefore, this chapter aimed to identify molecular mechanisms that could explain the

different behavioural and evolutionary, strategies of flight and infection in migratory Lepidoptera, using the fall armyworm (*Spodoptera frugiperda,* FAW) and *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV) as a model system.

The fall armyworm is a highly invasive, migratory crop pest. It is native to the Americas (North and South America) but spread to Africa in 2016 and by the end of 2018 it was confirmed as being present in 44 different African countries. In late 2018, it was confirmed as present in Asia, including in India and China, and in 2020 it was recorded in Australasia (FAO, 2021). Studying migration in this crop pest is highly important as it is a key factor in their rapid spread, with adults being able to fly up to 300 miles before mating and oviposition (Johnson, 1987).

SfMNPV occurs naturally in both the native and invasive range of FAW (Virgen et al., 2013; Lei et al., 2020). As well as occurring naturally, in the Americas and Australia SfMNPV can be used as a biocontrol mechanism (APVMA, 2021; Castillejos et al., 2002), and it is currently in the process of being registered for use in seven sub-Saharan African countries (Bateman et al., 2021). However, following exposure to SfMNPV some individuals can survive until adulthood but carry a covert, sub-lethal infection. Supporting the theory of Bateman's principle playing a role in migration and immunity, male FAW with covert SfMNPV infection had reduced flight capacity compared to non-infected males, with reduced flight durations and fewer flights resulting in infected individuals flying shorter distances, showing evidence of migratory culling (Pearson, 2016). In contrast, no change was observed in the flight capacity of females, suggesting that they were able to maintain their migratory potential when infected with the virus, undergoing migratory escape (Pearson, 2016).

To investigate the underlying molecular changes leading to these differing responses between the sexes, male and female fall armyworm larvae were exposed to a sub-lethal dose of SfMNPV and the adults were forced to fly for 5 hours. Following this, RNA was extracted and differential gene expression analysis was carried out using RNA-seq to identify changes between the sexes during flight in SfMNPV challenged and non-challenged individuals. Overall, this chapter provides a novel opportunity to investigate potential transcriptomic signatures of Bateman's principle to improve understanding of the underlying mechanisms behind these differences to enhance our knowledge of migration and disease.

4.2.1 Hypotheses

By using RNA-sequencing to identify gene expression changes in flown and not-flown FAW following SfMNPV challenge, this chapter focused on the underlying molecular mechanisms driving flight and immunity in a migratory crop pest to address the following hypotheses:

1. Gene expression will be similar in males and females in those areas most important for survival, such as metabolism and protein production.
2. Males and females will have different gene expression patterns in some aspects, specifically, it is hypothesised that observed transcriptomic changes will show that males invest more in reproduction and females in immunity.
    a. This will be observed through an upregulation of genes involved in reproduction in males and a downregulation, or no change, in genes involved in reproduction in females.
    b. This will be observed through an upregulation of genes involved in immunity in females and a downregulation, or no change, in genes involved in reproduction in males.

4.3 Methodology

4.3.1 Culture

Fall armyworm (*Spodoptera frugiperda,* FAW) larvae were collected by USDA ARS in Texas in October 2015. These were shipped to Rothamsted Research and raised to adults to create a starting population of 10 pairs. Larvae and adults were reared in 25°C, 14h light: 10h dark with no dusk/dawn simulation. Larvae were fed on an artificial diet (Appendix B, Table S3). Seventh generation adults were used in this experiment. Eggs were collected daily and transferred to a new rearing schedule at 25°C with 15h light: 9h dark, with a 30minute dusk or dawn simulation.

4.3.2 Viral challenge

In this experiment the focus was on the molecular differences between males and females during flight as previous work had shown sex dependent differences in flight behaviour in infected FAW (Pearson, 2016). Due to limited funds, and previous observations of differences in behaviour between the sexes, it was decided to invest in a larger number of treatment replicates at the expense of an uninfected control to increase the statistical validity of a male to female comparison. FAW larvae were challenged with the wild-type *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV). The SfMNPV was sourced from Nicaragua and provided by Departamento de Producción Agraria, Universidad Pública de Navarra, Pamplona, Spain. Four hundred fourth instar larvae were given 1µl $1x10^3$ OBs/µl SfMNPV on a diet plug in an optical well plate. Any individuals that did not eat the diet plug were discarded. Larvae were monitored daily until emergence.

4.3.3 Flight

On the day of emergence, adults were given cotton wool soaked in distilled water. They were then randomly assigned to either flown or not-flown treatments. The distilled water was removed from both treatment groups at the same time. Not-flown individuals were placed above the flight mills at the start of dusk and snap-frozen after five hours (Fig. 4.1). Flown individuals were randomly assigned a flight mill and attached to the flight mill handles following standard procedures (Jones et al., 2015; Pearson, 2016). All individuals were provided with a piece of paper to keep them stationary until dusk. At dusk, the paper was removed and they were forced to fly for five hours and then snap-frozen. When an individual attempted to stop flying they were gently prodded, if they did not respond after 3 prods they were assumed exhausted and removed.

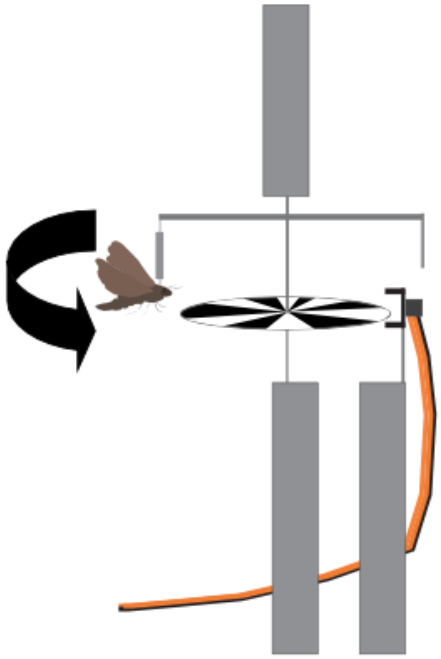


Figure 4.1 Flight mill. The moth is attached to a small pin using adhesive, and is then able to freely fly around the mill which is attached to a computer to record variables including speed, the number of flights, and distance.

4.3.4 RNA extraction and sequencing

RNA was extracted from 16 individuals from each of the four categories; male flown, male not-flown, female flown, female not-flown. For each treatment group four individuals were pooled together to provide sufficient RNA for extraction. RNA was then extracted following the Qiagen AllPrep DNA/RNA mini kit protocol. RNA next-generation sequencing was carried out by The Genome Analysis Centre (now Earlham Institute).

4.3.5 Processing RNA reads

Quality of reads was assessed using FastQC (Andrews, 2014), overall data was a good quality without adapter contamination. HISAT2 (Daehwan et al., 2015) was used to align paired reads to the corn genome annotation as the FAW used in this study were the corn strain (Gouin et al., 2017). HISAT2 was used as it was more stringent than other methods, for example higher alignment was found with BWA (Li and Durbin, 2009), however, further investigation showed that this aligned mitochondrial reads elsewhere on the genome when the mitochondrial genome was not provided whereas HISAT did not do this. The number of reads aligned to each part of the genome was calculated using featureCounts (Liao et al., 2014).

4.3.6 Differential expression analysis

A PCA plot was produced in DESeq2 to show the clustering of the samples based on gene expression (Love et al., 2014). Differential expression analysis was completed using DESeq2

in R (version 3.4.4) and default arguments including the interaction term were used for the deseq function. This function uses a negative binomial model to identify differentially expressed genes. The interaction term looks for significant differential expression between the sexes (Table 4.1). The default settings were used which included Cook's distance statistic to identify outliers and remove them from analysis. Blast2Go was used to identify gene sequences using cloudBlast and InterproScan to identify genes and GO terms (Conesa and Gtz, 2005). An enrichment analysis (Fisher's exact test) was carried out in Blast2Go (Conesa and Gtz, 2005) using an FDR <0.05.

Table 4.1: Differential expression analysis. The four potential outcomes of differential expression are described in the table below. Differential expression analysis compares the differences in fold-changes between flown and not-flown FAW of both sexes, and if there are interactions between males and females.

Differential expression outcomes	Fold change difference in a flown male	Fold change difference in a flown female
A. Differential expression in males and females. The fold change in expression is the same in both sexes. This can be upregulated or downregulated.	↑ / ↓	↑ / ↓
B. Differential expression in one sex. The fold change only changes in one sex, this can be upregulated or downregulated.	↑ / ↓	=
C. Differentially expressed genes showing an interaction between the sexes. The fold change is in significantly different directions for each sex. They can be upregulated or downregulated.	↑	↓
D. No change. There is no change in the genes expression in either sex.	=	=

4.4 Results

4.4.1 Females have more consistent gene expression patterns than males.

The PCA showed distinct clustering of two categories; females flown, females not-flown (Fig. 4.2). In contrast, in males, the not-flown and flown individuals did not appear to form their own clusters, or be closely associated with either of the other 3 groups. There are two male outliers which appear quite distinct from the other samples (Fig. 4.2). The differential expression analysis removed counts of individual genes that were likely to be outliers due to non-biological reasons (e.g., sequencing errors), suggesting a biological reason behind these flown male outliers.

During flight there were 742 differentially regulated genes compared to moths that were not-flown. The top 50 differentially expressed genes in each sex are shown in Fig. 4.3. The differential expression of 159 genes was shared by both males and females suggesting these were key for flight in both sexes, of these 53 were downregulated and 106 were upregulated. There were more differentially expressed genes in males compared to females; 100 downregulated genes and 257 upregulated genes, compared to 139 downregulated genes and 87 upregulated respectively.

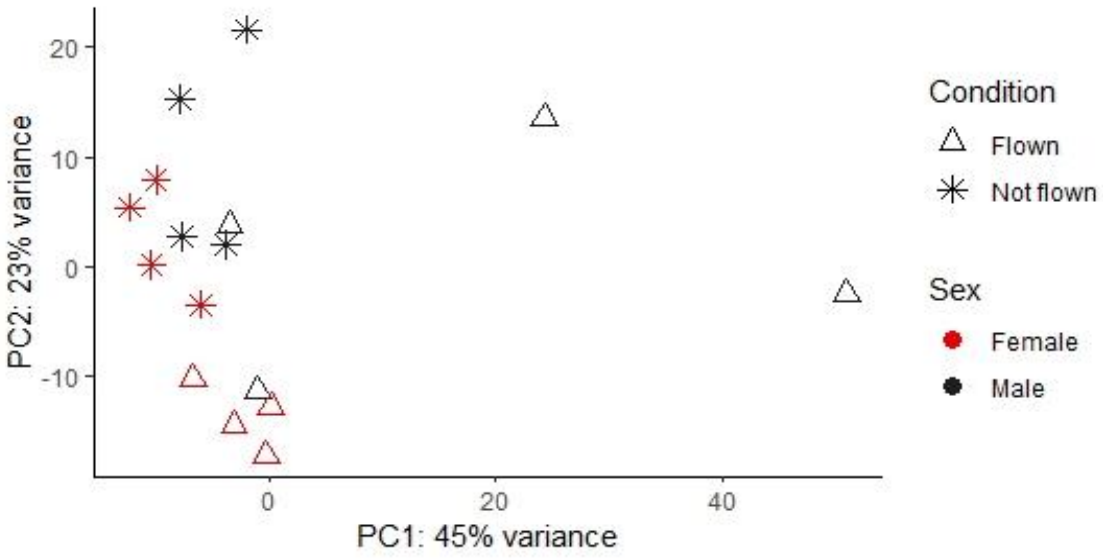


Figure 4.2 PCA plot showing clustering of samples. A principal components analysis (PCA) was carried out on the read counts, this showed distinct clustering of females not flown and flown, whereas males did not form such distinct clusters suggesting there is greater variation in males than females. Read counts are based on log2 transformed RNA-seq read counts.

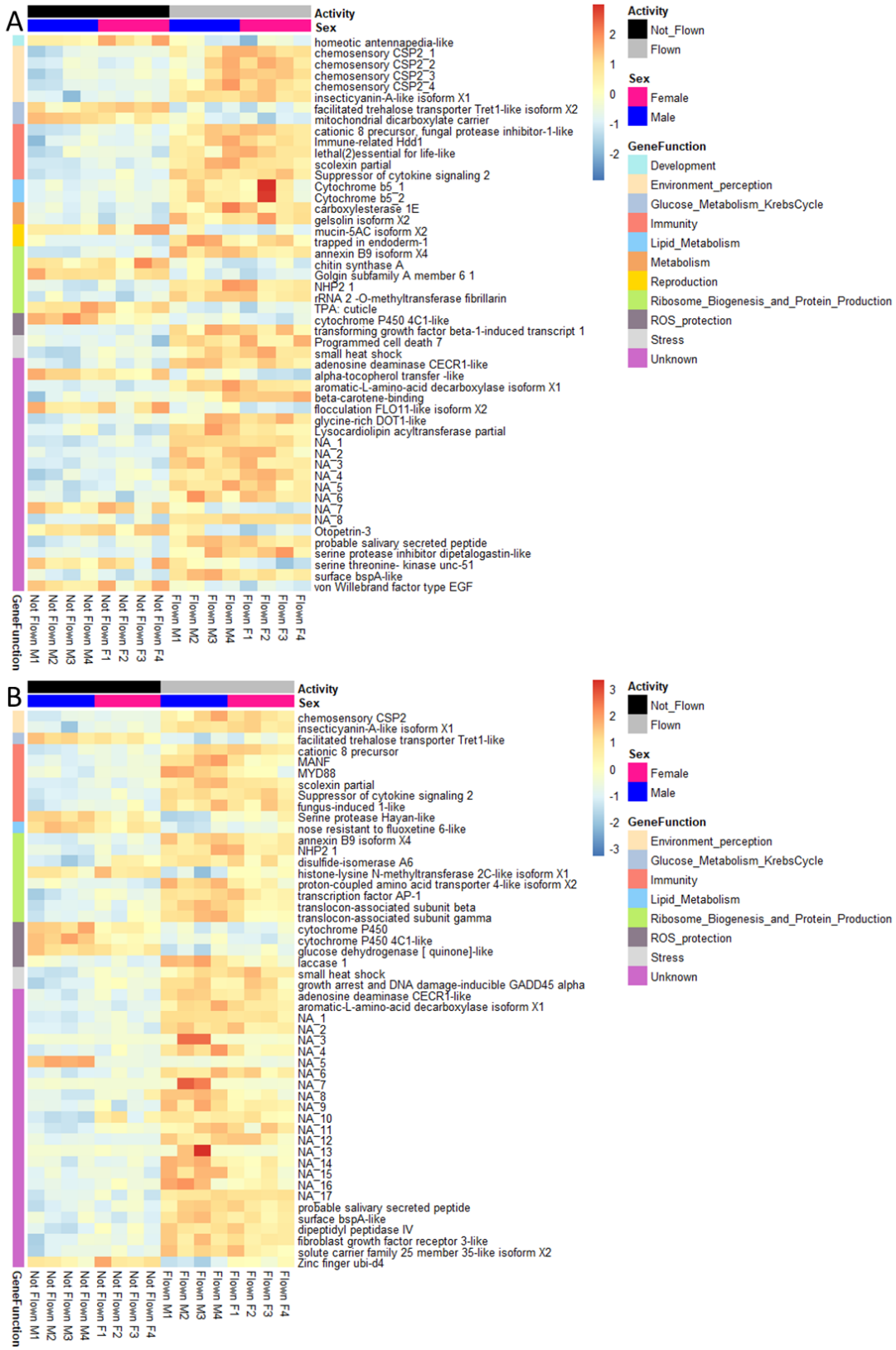


Figure 4.3 Top 50 differentially expressed genes in FAW. The fifty genes showing the greatest expression change between flown and not flown fall armyworm, for A) females and B) males.

4.4.2 Ten genes showed a significant interaction between sex and flight.

This study identified 10 genes that showed a significant interaction between males and females during flight, meaning that, for these genes, the fold-change is significantly different between males and females (Table 4.2). For seven of the differentially expressed genes, no further information on their function or role was available as they were hypothetical or uncharacterised proteins (Table 4.2).

Table 4.2: Genes showing a significant interaction between the sexes during flight. A significant interaction is defined as gene expression that was in significantly different directions between the sexes, however, the expression change within each sex is not always significant. *P* adjusted shows the *P* value following DeSeq analysis including the default correction for multiple tests.

Sequence Name	**Sequence Description**	**log2 fold change**	***P* adjusted**	**Female log2 fold change**	**Male log2 fold change**	**Annotation GO Term**
GSSPFG00020560001.3	*venom protease-like / clip-domain serine protease*	1.38	0.003	-2.45	N.S.	Proteolysis, serine-type endopeptidase activity
GSSPFG00018900001	*mucin-17*	1.34	0.049	N.S.	1.26	
GSSPFG00016587001.3	*MYD88 / Death domain-containing membrane NRADD*	1.44	0.020	N.S.	1.18	signal transduction, protein binding
GSSPFG00007203001.1	uncharacterized protein	9.43	0.020	N.S.	10.09	
GSSPFG00026782001	hypothetical protein	11.27	0.010	N.S.	8.40	
GSSPFG00022386001	uncharacterized protein	24.11	0.014	N.S.	23.73	
GSSPFG00023594001	NA	-10.13	<0.001	N.S.	-7.91	
GSSPFG00009482001	NA	9.44	0.005	N.S.	10.42	
GSSPFG00009733001	NA	0.74	0.014	N.S.	0.79	
GSSPFG00023536001	NA	1.98	0.015	N.S.	2.10	

Flight caused the expression of a clip-domain serine protease to have a significantly different response in the two sexes, i.e., flight led to lower gene expression in males and higher gene expression in females (Fig. 4.4a). Based on known genes in *Bombyx mori*, the function of this clip-domain serine protease is to cleave peptide bonds that could initiate the Toll-pathway (Cheng et al., 2016a). There was further evidence that males downregulate serine proteases during flight, with two others downregulated and one serine protease inhibitor upregulated, suggesting that males may have been reducing the levels of serine proteases during flight. In contrast, no serine proteases were downregulated in females. A serine protease inhibitor dipetalogastin-like was upregulated in both males and females during flight. However, the function of this inhibitor in Lepidoptera is unknown, as it is identified from the triatomid bug, *Dipetalogaster maximus*, in which it functions to prevent blood clotting to allow them to feed on blood (Mende et al., 2001).

There was a significant interaction between the sexes in the expression of MYD88, with MYD88 being strongly upregulated during flight in males, whilst it was either downregulated or only slightly upregulated in females (Fig. 4.4b). MYD88 has a role in the immune system; following Spätzle activation, the active Toll receptor forms a signalling-complex including MYD88. This results in a signalling cascade that triggers the transcription of target genes such as antimicrobial peptides (Valanne et al., 2011).

Mucin-17 was upregulated in males during flight, whereas expression levels barely changed between flown and not-flown females (Fig. 4.4c). However, another mucin-17 gene was significantly downregulated in both sexes during flight. The primary function of mucins in vertebrates is to protect internal organs by contributing to the formation of a barrier. In Drosophila, 42 mucin-like proteins have been identified, which appear to have a wide variety of roles such as in embryonic development and in adults they localised to cuticle free organs which would suggest they share a similar protective function (Syed et al., 2008).

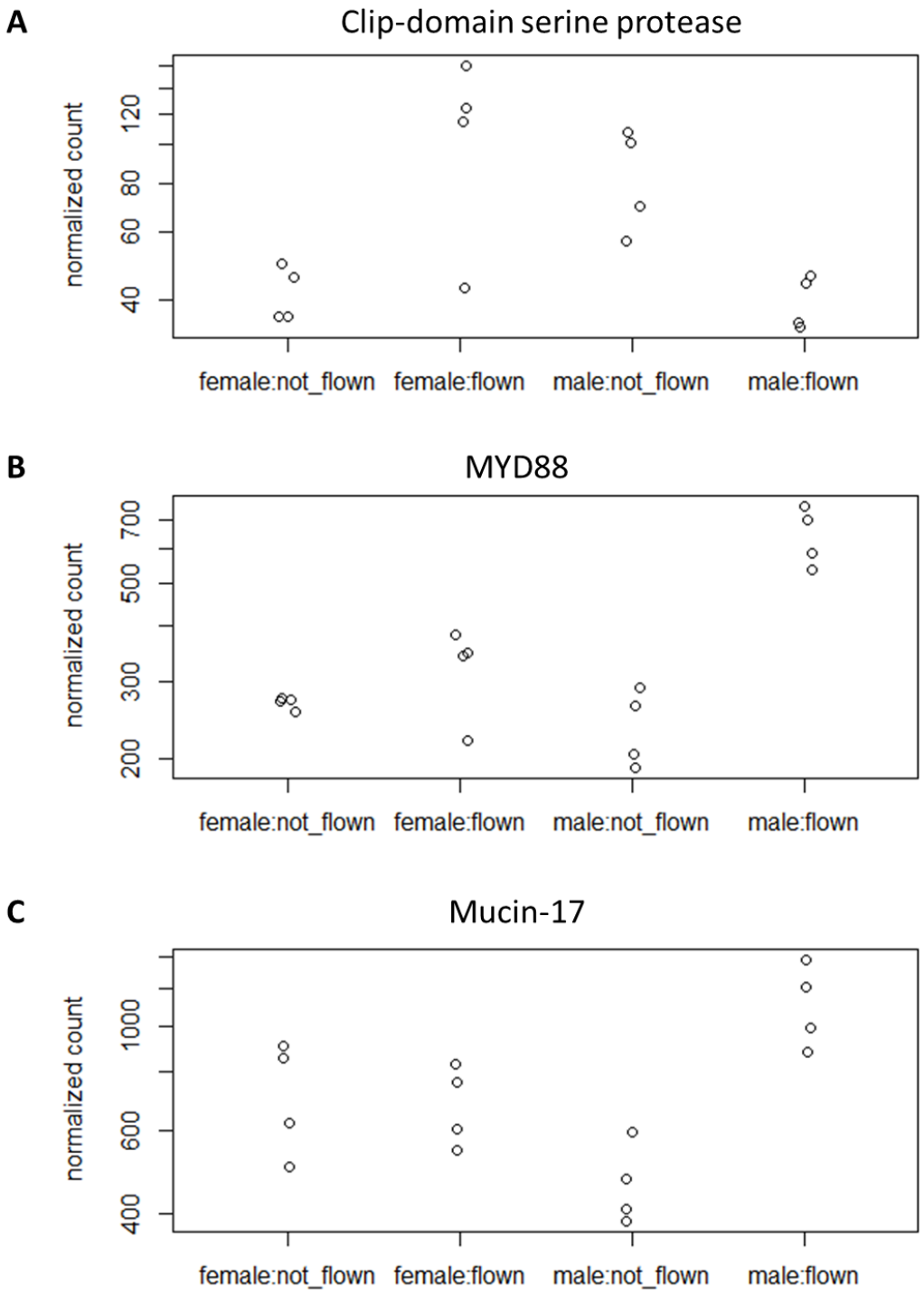


Figure 4.4 Gene counts of genes showing a significant interaction between the sexes. The significant interaction shows that the response the gene was in significantly different directions in each sex, this can be upregulated, downregulated or no response in one sex whilst the other sex showed a significant response. Counts of the three genes that showed significant differential expression between the sexes during flight. A) *Clip-domain serine protease* B) *MYD88* and C) *Mucin 17*.

4.4.3 Immune related genes were upregulated in both sexes during flight.

There were many immune related genes differentially expressed during flight in both sexes, including two previously discussed in section 4.3.1 (clip-domain serine protease and MYD88) that showed significant differential expression between the sexes.

There were multiple components of the Toll-pathway differentially expressed during flight in both sexes. Interestingly, two components were significantly differently expressed between the sexes; *clip-domain serine protease* expression increased significantly during flight in females compared to males, and *MYD88* expression significantly increased in flight in males compared to females. Both *cactus* and *defensin* expression significantly increased during flight in both sexes. The clip-domain serine protease may be involved in initiating the Toll-pathway cascade (Fig. 4.5). After initiation, MYD88 binds to the Toll receptor and triggers the formation of a complex of proteins, resulting in a signalling cascade that releases cactus which is consequently broken down. This results in the release of a transcription factor (DIF) that tiggers the expression of antimicrobial peptides (Fig. 4.5).

Other genes with potential roles in immunity that were upregulated during flight in both sexes include Hdd1, Hdd23 and MANF (Fig. 4.6). A summary of the immune-related genes differentially expressed during flight is provided in Table 4.6.

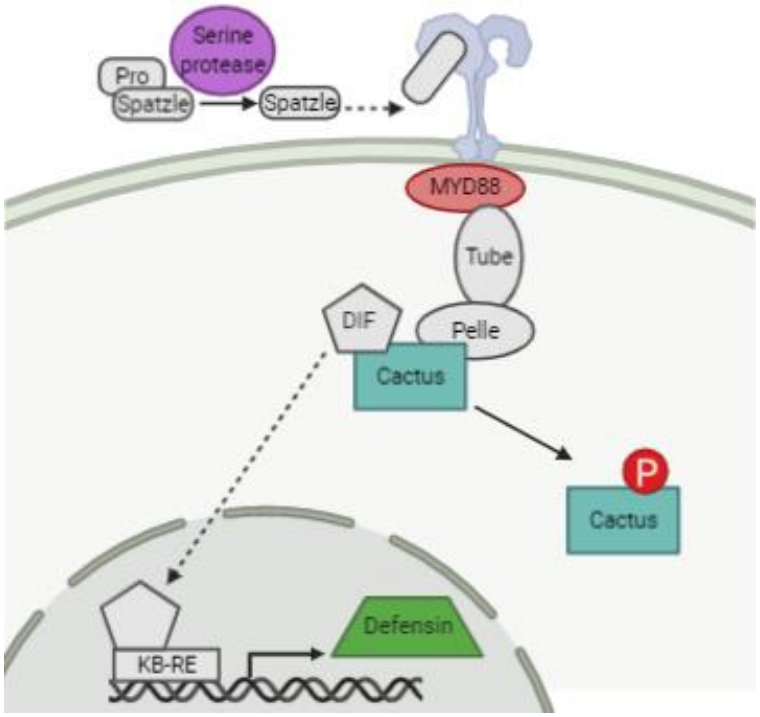


Figure 4.5 Components of the Toll-pathway differentially expressed during flight in FAW. These were clip-domain *serine protease, MYD88, cactus* and *defensin*. *Serine protease* expression increased significantly during flight in females compared to males. *MYD88* expression significantly increased in flight in males compared to females. Both *cactus* and *defensin* expression significantly increased during flight in both sexes. Image created in BioRender© 2021.

4.4.4 Genes involved in protein production were upregulated during flight in both sexes.

In the set of genes that were upregulated during flight in both males and females, there was an overrepresentation of gene ontology (GO) terms related to ribosomes and protein production, meaning that the expression of many genes related to these functions increased during flight (Table 4.3). Additionally, six transcription factors in males and five transcription factors in females that were associated with protein synthesis were upregulated in response to flight, including those with roles in ribosome production and translation initiation. Furthermore, in both males and females, genes for enzymes associated with aminoacid-tRNA biosynthesis were upregulated (Fig 4.6, Table 4.6).

Further evidence of increases in production of proteins due to flight comes from the overrepresentation of genes in males linked to the endoplasmic reticulum, which functions in the synthesis of proteins and lipids (Table 4.3). Additionally, three *box H/ACA ribonucleoproteins* were found to be upregulated in males and two in females (Fig 4.6, Table 4.6). These *box H/ACA ribonucleoproteins* are involved in the posttranscriptional pseudouridylation of RNA, which is important for RNA function as a loss of pseudourins can lead to inefficient splicing (Hamma and Ferré-D'Amaré, 2010; De Zoysa and Yu, 2017).

This study identified genes associated with endoplasmic reticulum stress and the removal of incorrectly folded proteins in both sexes (Fig 4.6, Table 4.6). *MANF* was upregulated which plays a role in reducing endoplasmic reticulum stress by increasing the unfolded protein response which increases the folding capacity of chaperones and enhances the degradation of incorrectly or unfolded proteins (Lindstrom et al., 2016). In females, *X-box-binding 1* was upregulated during flight, which plays a role in responding to and removing unfolded proteins (Chen et al., 2012). This signalling pathway is conserved across many taxa and works via activation of the Ire-1 pathway and is important in aiding cell recovery after endoplasmic reticulum stress (Plongthongkum et al., 2007).

The *nucleosome-remodelling factor subunit NURF301* was downregulated in females (Fig 4.6, Table 4.6). *NURF301* is sequence specific so targets specific genes and is essential for nucleosome sliding for transcription so is involved in regulating transcription and chromatin structure (Xiao et al., 2001; Alkhatib and Landry, 2011). It is ATP dependent so its downregulation could be a mechanism of ensuring more ATP is available for flight. Alternatively, *NURF301* may be specific to genes that are not needed during flight.

Table 4.3 GO terms related to ribosome biogenesis and protein production that were overrepresented during flight. The GO (Gene Ontology) terms that are most common amongst the differentially expressed genes, this can give an indication of areas where most gene expression changes are occurring.

Expression	Sex	GO term	Function
↑	M	endoplasmic reticulum	Endoplasmic reticulum - protein production
↑	M	endoplasmic reticulum membrane	Endoplasmic reticulum - protein production
↑	M	nuclear outer membrane-endoplasmic reticulum network	Endoplasmic reticulum - protein production
↑	M	endoplasmic reticulum part	Endoplasmic reticulum - protein production
↑	M	endoplasmic reticulum sub-compartment	Endoplasmic reticulum - protein production
↑	M	box H/ACA snoRNP complex	Pseudouridylation - RNA function
↑	M	box H/ACA RNP complex	Pseudouridylation - RNA function
↑	Both	ribonucleoprotein complex biogenesis	Ribosome biogenesis - RNA translation
↑	Both	ribosome biogenesis	Ribosome biogenesis - RNA translation
↑	Both	cellular component biogenesis	Ribosome biogenesis - RNA translation
↑	F	ribosome	Ribosome biogenesis - RNA translation
↑	F	tRNA metabolic process	Ribosome biogenesis - RNA translation
↑	F	translation	Ribosome biogenesis - RNA translation
↑	F	peptide biosynthetic process	Ribosome biogenesis - RNA translation
↑	M	small nucleolar ribonucleoprotein complex	Ribosome biogenesis - RNA translation
↑	M	rRNA processing	Ribosome biogenesis - RNA translation
↑	M	protein N-linked glycosylation	Ribosome biogenesis - RNA translation
↑	M	rRNA metabolic process	Ribosome biogenesis - RNA translation

4.4.5 Flight increased energy metabolism genes in females and decreased them in males; gene expression suggests that flight is fuelled by carbohydrates in females and lipids in males.

In males, there was overrepresentation of GO terms relating to metabolism in the downregulated genes (Table 4.4). In contrast, in females there was upregulation of genes associated with GO terms linked to metabolic processes such as the processing of carboxylic acid; this processing is important for energy production via the Krebs cycle (Table 4.4).

In females there was the upregulation of three enzymes that are part of the Krebs and TCA cycle; *dehydrogenase (NAD+), succinyltransferase, and aconitase* (Fig 4.6, Table 4.6). *Aconitase* is important in the TCA cycle and catalyses the conversion of citrate to isocitrate. In *Drosophila melanogaster,* reduced expression of *aconitase* resulted in decreased locomotor activity and the TCA cycle was impaired resulting in increased lipid metabolism and a shortened lifespan (Cheng et al., 2013). This supports evidence from the GO terms that there was an overrepresentation of carboxylic acid processing genes that are linked to the Krebs cycle. However, none of these GO terms or enzyme pathways associated with the Krebs cycle were significantly differentially expressed in males (Table 4.4, Fig. 4.6, Table 4.6).

Anamorsin, a negative regulator of apoptosis, was upregulated in both sexes (Fig 4.6, Table 4.6). *Anamorsin* has been found to have a range of functions, including one in metabolism and one in immunity. Firstly, it is necessary for the early stages of the iron-sulphur cluster assembly and acts as an electron transfer protein which can accept mitoNEET and NAF-1 when oxidised, which are both found on the outer membrane of the mitochondria (Lipper et al., 2015). These iron-sulphur clusters are important in oxidation-reduction processes during metabolism. Secondly, in response to some cytokines *anamorsin* can lead to cell survival by inhibiting apoptosis (Shibayama et al., 2004) and it has been linked to the immune response by inhibiting apoptosis linked to pathogenic stress in the rock bream (*Oplegnathus fasciatus*) (Elvitigala et al., 2015).

Table 4.4 GO terms related to metabolism that were overrepresented during flight. The GO (Gene Ontology) terms that are most common amongst the differentially expressed genes, this can give an indication of areas where most gene expression changes are occurring.

Expression	Sex	GO Name	Function
↑	F	organic acid metabolic process	Carboxylic acid metabolism, Krebs cycle
↑	F	cellular amino acid metabolic process	Carboxylic acid metabolism, Krebs cycle
↑	F	carboxylic acid metabolic process	Carboxylic acid metabolism, Krebs cycle
↑	F	oxoacid metabolic process	Carboxylic acid metabolism, Krebs cycle
↑	F	cellular amide metabolic process	Carboxylic acid metabolism, Krebs cycle
↑	F	amide biosynthetic process	Carboxylic acid metabolism, Krebs cycle
↑	F	peptide metabolic process	Metabolic process
↑	F	small molecule metabolic process	Metabolic process
↓	M	monooxygenase activity	Metabolic process
↓	M	oxidoreductase, acting on paired donors, with incorporation or reduction of molecular oxygen	Metabolic process
↓	M	oxidation-reduction process	Metabolic process

Reactive oxygen species (ROS) are produced as a by-product of energy metabolism and have been shown to increase during flight (Syromyatnikov et al., 2015). In males there were four GO terms related to ROS protection overrepresented in upregulated genes (Table 4.5).

Glutathione metabolism is involved in protecting the body from reactive oxygen species (Dringen, 2000). In both sexes, there was upregulation of the glutathione metabolism pathway involving the enzymes decarboxylase, transferase, and dehydrogenase (NADP+-dependent, decarboxylating). In males, two *glutathione S-transferases* (*GST*), including *GST omega 1*, were upregulated during flight (Fig. 4.6, Table 4.6). *GST* has been linked to protecting against neuronal damage that could be caused by oxidative stress in *D. melanogaster,* with *GST omega 1* mutant flies being susceptible to ROS, leading to neuronal damage and death, which could be protected by neuron-specific expression of *GST omega 1* (Kim et al., 2017).

Transforming growth factor beta-1-induced transcript 1 (*TGF-β1*) was upregulated in both sexes. There is some evidence that *TGF-β1* is activated by ROS and potentially helps to regulate and reduce oxidative stress (Barcellos-Hoff and Dix, 1996).

An intermediate product of reactive oxygen species during aerobic metabolism is hydrogen peroxide, which is particularly reactive with iron. Due to this iron regulatory proteins are key in the management of oxidative stress and are often upregulated as ROS increases (Pantopoulos and Hentze, 1995). In females, the enzyme *ferroxidase* was upregulated which is essential for iron homeostasis in *D. melanogaster (Lang et al., 2012)*.

Table 4.5 GO terms related to ROS protection that were overrepresented during flight. The GO (Gene Ontology) terms that are most common amongst the differentially expressed genes, this can give an indication of areas where most gene expression changes are occurring.

Expression	Sex	GO Name	Function
↑	M	cellular homeostasis	ROS stress
↑	M	homeostatic process	ROS stress
↑	M	cell redox homeostasis	ROS stress
↑	M	regulation of biological quality	ROS stress

In both males and females, two enzymes involved in galactose metabolism were upregulated: *epimerase* and *invertase* (Fig 4.6, Table 4.6). This suggests that glycolysis was one of the processes used to provide the energy needed for flight in both species.

Usually during prolonged flight in insects metabolism switches from using carbohydrates as fuel to lipids (Wegener, 1996; Jones et al., 2015; Wang et al., 2020). There was evidence of

lipid metabolism in males whereas evidence of this was limited in females; in males, two lipases were downregulated (*lipase 3-like* and *phospholipase A-2-activating*) whilst four were upregulated (Fig 4.6, Table 4.6). The upregulated lipase genes included a *secretory phospholipase A2 receptor-like* and *an 85-88 kDa calcium-independent phospholipase A2* (Fig 4.6, Table 4.6). Phospholipase A2 enzymes have been associated with lipid mobilisation for the breakdown of lipids for lipid-fuelled flight (Arrese and Soulages, 2010; Jones et al., 2015). Alternatively, there is some evidence that phospholipase A2 enzymes may have an important role in immunity, in the beet armyworm (*Spodoptera exigua*), RNAi treatment of a calcium-independent phospholipase A2 gene resulted in supressed immunity (Sadekuzzaman et al., 2017). Although no lipases were found to be upregulated in females, *inositol-trisphosphate 3-kinase A isoform X1* was upregulated which is linked to lipid signalling via the inactivation of fatty acid synthesis (Fig 4.6, Table 4.6).

4.4.6 Flight increased reproduction-related genes in males and decreased them in females.

Two *forkhead box L2-like isoform X2* (*FOXL2*) genes were overexpressed in flown males (Fig 4.6, Table 4.6), this gene was essential for normal development of the testes in zebra fish (*Danio rerio*) (Webster et al., 2017).

A gene identified as *SOX-9-like* was found to be upregulated in males during flight (Fig. 4.6, Table 4.6), SOX-9 is a vertebrate gene but the invertebrate gene most similar is the SoxE group (Crémazy et al., 2001). In *D. melanogaster* the *SoxE* gene is essential for testes development during the pupal stages, with mutants unable to express *SoxE* found to have severely reduced or absent testes, and reduced pigmentation in the testes (Nanda et al., 2009).

In females, *mucin 5AC isoform 1* and *mucin 5AC isoform 2* were both significantly downregulated during flight (Fig. 4.6, Table 4.6). This gene was found to be significantly upregulated in eggs in the Asian corn borer (*Ostrinia furnacalis*) which could suggest a potential role in egg development (Zhang et al., 2016).

Additionally, *shuttle craft* was downregulated during flight in females (Fig. 4.6, Table 4.6). *Shuttle craft* is required for development in embryonic nervous systems in drosophila so is expressed at higher levels in the ovaries (Stroumbakis et al., 1996). However, reducing expression of *shuttle craft* has been linked to increased longevity and activity in unmated female drosophila (Pasyukova et al., 2004; Roshina et al., 2014).

4.4.7 Flight led to the downregulation of genes involved in the circadian rhythm.

The circadian clock gene *CLOCK* (*circadian locomotor output cycles kaput-like*) was downregulated in flying females during this experiment (Fig. 4.6, Table 4.6). In moths (*Operophtera brumata* and *Spodoptera litura*), the circadian clock genes *per*, *tim* and *cyc2* are downregulated during the day and upregulated at night. *Per* and *cyc2* bind together to form a dimer which inhibits the transcription of *CLOCK* and *CYCLE* thus the downregulation of *CLOCK* at night is expected (Karthi and Shivakumar, 2014; Derks et al., 2015). There was the downregulation of a *circadian-clock controlled* gene in both sexes, and another in just males during flight further supporting the evidence of flight triggering changes to the circadian rhythm (Fig. 4.6, Table 4.6).

The transcription factor *U2af50* (*U2 small nuclear riboprotein auxiliary factor 50*) was significantly downregulated during flight in both sexes (Fig. 4.6, Table 4.6). *U2af50* is considered to play a role in circadian rhythms in honeybees (*Apis spp.*) and fruit flies (*Drosophila melanogaster*) as downregulation by RNAi increased peak locomotor activity at dawn, however the amount of time spent inactive was not affected by RNAi of *U2af50* (Fu and Whitfield, 2012).

Figure 4.6 Heat map showing the expression of selected genes that were differentially expressed during flight in males, females or in both sexes. Further information on the genes included in this figure is provided in the corresponding sections above and in Table 4.6.

Table 4.6 Key genes related to immunity, reproduction, metabolism, ROS protection, protein production and the circadian rhythm that were significantly differentially expressed. These genes were differentially expressed in one, or both, sexes when fall armyworm infected with SfMNPV were flown. *P* shows the adjusted *P* value following the default correction for multiple testing carried out in DeSeq analysis in R. References explaining the potential function of the identified gene are provided.

Gene	Sequence name	Proposed function	Female		Male		Function references
			log2 fold change	*P*	log2 fold change	*P*	
Genes with a proposed function in immunity							
Suppressor of cytokine signalling 2 (SOCS2)	GSSPFG00006560001	Regulated by the Toll-pathway and Jak/Stat-pathway, increased expression of antimicrobial peptides, negative feedback loop of Toll-pathway	1.99	<0.001	2.62	<0.001	(Letellier and Haan, 2016), (Sun et al., 2016), (Posselt et al., 2011), (Schwarz et al., 2013)
mesencephalic astrocyte-derived neurotrophic factor homolog (MANF)	GSSPFG00003037001	Expressed in response to bacteriocytes and co-localises with Toll-like receptor	0.66	0.03	1.68	<0.001	(Sereno et al., 2017)
beta-1,3-glucan-binding -like	GSSPFG00019060001.5	Recognise bacteria/fungi, prophenoloxidase cascade, encapsulation, expression of antimicrobial peptides, upregulated in response to virus	0.91	0.02	1.09	<0.001	(Wang et al., 2005), (Kim et al., 2000), (Roux et al., 2002)
beta-1,3-glucan-binding -like	GSSPFG00000179001.3	As above	1.30	<0.001	NA	NS	As above
Hdd1	GSSPFG00027601001.2	Increased expression in response to bacterial infection	1.91	<0.001	2.25	<0.001	(Woon Shin et al., 1998), (Zhang et al., 2017)
Hdd1	GSSPFG00026567001.2	As above	NA	NS	2.25	0.04	As above
Hdd23	GSSPFG00007232001	Upregulation in response to bacterial infection, up and down regulation in response to viral infection reported in *Spodoptera spp.*	2.32	0.01	2.31	0.01	(Woon Shin et al., 1998), (Jakubowska et al., 2013), (Provost et al., 2011)

Clip domain serine protease	GSSPFG00020560001.3	May initiate the Toll-pathway cascade, or be involved in melanisation	1.38	0.004	NA	NS	(Valanne et al., 2011; Kanost and Jiang, 2015)
Serine protease easter-like	GSSPFG00005796001	May initiate the Toll-pathway cascade					(Valanne et al., 2011; Kanost and Jiang, 2015)
MYD88	GSSPFG00016587001.3	Component of the Toll-induced signalling complex (TISC)	NA	NS	1.44	<0.001	(Valanne et al., 2011)
Cactus	GSSPFG00001145001.2	Component of Toll-induced signalling complex (TISC) that is degraded to release transcription factors	1.03	0.030	1.39	0.002	(Valanne et al., 2011)
Cactus	GSSPFG00035666001.2	Component of Toll-induced signalling complex (TISC) that is degraded to release transcription factors	NA	NS	1.21	<0.001	(Valanne et al., 2011)
Defensin	GSSPFG00008360001.3	An antimicrobial peptide	1.60	0.002	2.18	<0.001	(Xi et al., 2008; Dong et al., 2016; Lourenço et al., 2018)
Genes with a proposed function in reproduction							
FOXL2	GSSPFG00001837001	Testes development	NA	NS	1.98	0.033	(Webster et al., 2017)
FOXL2	GSSPFG00014276001.2	Testes development	NA	NS	2.26	0.043	(Webster et al., 2017)
SOX-9	GSSPFG00032712001	Testes development	NA	NS	3.17	0.017	(Nanda et al., 2009)
mucin-5AC_X1	GSSPFG00027357001	Egg development	-0.90	0.012	NA	NS	(Zhang et al., 2016)
mucin-5AC_X2	GSSPFG00021557001	Egg development	-1.09	0.001	NA	NS	(Zhang et al., 2016)
mucin-5AC-like_X1	GSSPFG00034469001	Egg development	-1.09	0.018	NA	NS	(Zhang et al., 2016)
shuttlecraft	GSSPFG00010709001.3	Egg development	-0.73	0.043	NA	NS	(Stroumbakis et al., 1996)
Genes with a proposed function in metabolism							
anamorsin	GSSPFG00030302001	Oxidation-reduction process during metabolism. Also potentially has an immune function to inhibit apoptosis.	1.06	0.012	1.28	0.001	(Shibayama et al., 2004; Lipper et al., 2015)
epimerase	GSSPFG00011867001	Galactose Metabolism	1.04	0.024	1.61	<0.001	(Götz et al., 2008; Kanehisa Laboratories, 2021)

invertase	GSSPFG00014912001	Galactose Metabolism	0.84	0.006	1.14	<0.001	(Götz et al., 2008; Kanehisa Laboratories, 2021)
aconitase	GSSPFG00029957001.1	Glucose Metabolism / Krebs Cycle	1.20	0.039	NA	NS	(Cheng et al., 2013)
isocitrate dehydrogenase [NAD]_mitochondrial isoformX2	GSSPFG00032424001	Glucose Metabolism / Krebs Cycle	2.09	0.04	NA	NS	(Götz et al., 2008; Kanehisa Laboratories, 2021)
succinyl transferase	GSSPFG00011006001	Glucose Metabolism / Krebs Cycle	1.61	0.03	NA	NS	(Götz et al., 2008; Kanehisa Laboratories, 2021)
lipase3-like	GSSPFG00012548001	Lipid Metabolism	NA	NS	-16.15	0.006	(Götz et al., 2008; Kanehisa Laboratories, 2021)
phospholipase A-2-activating	GSSPFG00000692001	Lipid Metabolism	NA	NS	-0.68	0.017	(Arrese and Soulages, 2010; Jones et al., 2015)
lipase member H-B-like	GSSPFG00004803001	Lipid Metabolism	NA	NS	1.26	0.008	(Götz et al., 2008; Kanehisa Laboratories, 2021)
secretory phospholipase A2 receptor-like	GSSPFG00035412001.4	Lipid Metabolism	NA	NS	1.51	0.036	(Arrese and Soulages, 2010; Jones et al., 2015)
8588kDa calcium-independent phospholipase A2	GSSPFG00001395001	Lipid Metabolism	NA	NS	2.02	0.027	(Arrese and Soulages, 2010; Jones et al., 2015)
lipase member H-A-like	GSSPFG00020385001	Lipid Metabolism	NA	NS	3.69	0.021	(Götz et al., 2008; Kanehisa Laboratories, 2021)
inositol-trisphosphate3-kinase A isoformX1	GSSPFG00013047001	Lipid Metabolism	1.73	0.046	NA	NS	(Götz et al., 2008; Kanehisa Laboratories, 2021)

Genes with a proposed function in Reactive Oxygen Species (ROS) protection							
ornithine decarboxylase	GSSPFG00025292001	ROS protection	2.84	0.010	NA	NS	(Götz et al., 2008; Kanehisa Laboratories, 2021)
Phosphogluconate dehydrogenase	GSSPFG00022033001	ROS protection	1.32	0.047	NA	NS	(Kim et al., 2017)
glutathioneS-transferaseomega1	GSSPFG00020910001.3	ROS protection	NA	NS	0.77	0.020	(Kim et al., 2017)
glutathioneS-transferase2-like	GSSPFG00022085001.3	ROS protection	NA	NS	4.57	0.050	(Kim et al., 2017)
ferroxidase	GSSPFG00028349001	ROS protection	0.92	0.048	NA	NS	(Götz et al., 2008; Kanehisa Laboratories, 2021)
transforming growth factor beta-1-induced transcript 1	GSSPFG00024425001.3	ROS protection	1.30	0.001	1.51	<0.001	(Barcellos-Hoff and Dix, 1996)
transforming growth factor beta-1-induced transcript 1	GSSPFG00021500001.3	ROS protection	NA	NS	1.42	<0.001	(Barcellos-Hoff and Dix, 1996)
Genes with a proposed function in ribosome biogenesis and protein production							
NURF301	GSSPFG00013452001	Ribosome biogenesis and protein production	-0.74	0.029	NA	NS	(Xiao et al., 2001; Alkhatib and Landry, 2011)
X-box-binding1	GSSPFG00010286001	Ribosome biogenesis and protein production	0.94	0.015	NA	NS	(Chen et al., 2012)
arginine tRNA ligase	GSSPFG00008374001	Ribosome biogenesis and protein production	0.74	0.20	0.88	0.003	(Götz et al., 2008; Kanehisa Laboratories, 2021)
methionine tRNA ligase	GSSPFG00007278001	Ribosome biogenesis and protein production	1.05	0.041	NA	NS	(Götz et al., 2008; Kanehisa Laboratories, 2021)
serine tRNA ligase	GSSPFG00026270001	Ribosome biogenesis and protein production	0.91	0.008	NA	NS	(Götz et al., 2008; Kanehisa Laboratories, 2021)

serine tRNA ligase	GSSPFG00016645001	Ribosome biogenesis and protein production	1.25	0.028	NA	NS	(Götz et al., 2008; Kanehisa Laboratories, 2021)
Threonine tRNA ligase	GSSPFG00021747001	Ribosome biogenesis and protein production	1.14	0.029	1.21	0.02	(Götz et al., 2008; Kanehisa Laboratories, 2021)
Threonine tRNA ligase	GSSPFG00004785001	Ribosome biogenesis and protein production	NA	NS	1.28	0.034	(Götz et al., 2008; Kanehisa Laboratories, 2021)
box H/ACA ribonucleoproteins	GSSPFG00023136001	box H/ACA ribonucleoproteins	NA	NS	1.42	0.023	(Hamma and Ferré-D'Amaré, 2010; De Zoysa and Yu, 2017)
box H/ACA ribonucleoproteins	GSSPFG00023366001	box H/ACA ribonucleoproteins	0.79	0.008	0.85	0.003	(Hamma and Ferré-D'Amaré, 2010; De Zoysa and Yu, 2017)
box H/ACA ribonucleoproteins	GSSPFG00010031001	box H/ACA ribonucleoproteins	NA	NS	1.52	<0.001	(Hamma and Ferré-D'Amaré, 2010; De Zoysa and Yu, 2017)
box H/ACA ribonucleoproteins	GSSPFG00014223001	box H/ACA ribonucleoproteins	NA	NS	1.02	0.003	(Hamma and Ferré-D'Amaré, 2010; De Zoysa and Yu, 2017)
Genes with a proposed function in the circadian rhythm							
Circadian clock-controlled	GSSPFG00000246001	Circadian rhythm	-1.11	0.011	-1.51	<0.001	(Götz et al., 2008)
Circadian clock-controlled	GSSPFG00004892001	Circadian rhythm	NA	NS	-1.68	<0.001	(Götz et al., 2008)
CLOCK	GSSPFG00005153001.3	Circadian rhythm	-0.76	0.034	NA	NS	(Karthi and Shivakumar, 2014; Derks et al., 2015)
splicing factor U2AF 50 kDa subunit	GSSPFG00005724001	Circadian rhythm	-0.42	0.028	-0.41	0.034	(Fu and Whitfield, 2012)

4.5 Discussion

Many genes were differentially expressed in SfMNPV-challenged FAW that were flown compared to those that were not-flown. Here, the implications of this differential expression and the potential driving mechanisms are discussed for the four key areas of interest: immunity, protein production, energy metabolism and reproduction.

4.5.1 Immunity

Immune differences between the sexes are most likely to be due to the different life histories and genetics of males and females that is driven by Bateman's principle (Bateman, 1948; Rolff, 2002). It has been proposed that if immune differences were driven by Bateman's principle in insects then females would have higher immunity than males (Bateman, 1948; Rolff, 2002), and it was hypothesised in this study that FAW females would show an upregulation of immune related genes compared to males. However, there was no strong evidence in our results to support the theory of increased immunity in females. Nevertheless, two of the genes that were significantly differentially expressed between the sexes were both related to the Toll-pathway, suggesting that there were some differences in immunity between the sexes.

Clip-domain serine proteases have a role in the activation of the Toll-pathway. This is via activating the cytokine Spätzle to form an active Toll ligand which triggers the synthesis of antimicrobial peptides (Kanost and Jiang, 2015). Furthermore, the upregulation of serine proteases is linked to immune responses in other insects, for example, gene expression of serine proteases increased in honey bees (*Apis mellifera*) infected with foulbrood bacteria (*Paenibacillus larva*) (Zou et al., 2006). However, some serine proteases increased in response to saline injections so their relationship in immunity was hard to determine as this could suggest that the upregulation was a general stress response (Zou et al., 2006). In this study, SfMNPV was given to larvae on diet plugs and FAW that were not-flown did not show an upregulation of clip-domain serine proteases, therefore it is more likely that the clip-domain serine proteases were upregulated as a response to infection and flight.

There is a lot of evidence that serine proteases play an important role in immunity in Lepidoptera to protect them against NPV infections. Fifth instar silkworm (*B. mori*) larvae had 100% survival rate when infected with *Bombyx mori nucleopolyhedrovirus* occlusion body derived (BmNPV-OD) and treated with the purified serine protease BmSP-2, compared to 100% mortality without this treatment (Nakazawa et al., 2004). Similarly, the Chinese oak silk moth (*Antheraea pernyi*) upregulated a trypsin-like serine protease during infection with

each of *Antheraea pernyi nucleopolyhedrovirus* (ApNPV*), Nosema pernyi, Enterococcus pernyi*, and *Beauveria bassiana*, with the greatest upregulation observed following infection with ApNPV (Sun et al., 2017). Together, these findings support the evidence from this work that serine proteases may be important in immunity against NPV viruses in Lepidoptera.

Both sexes showed clear evidence of the upregulation of the Toll-pathway during flight, including *MYD88, cactus* and *defensin*. The degradation of *cactus* is an important step in the Toll-pathway as this leads to the release of DIF which consequently induces the expression of antimicrobial peptides (Belvin and Anderson, 1995; Ferrandon et al., 2007). However, as *cactus* is a negative regulator of the Toll-pathway then its upregulation may inhibit the Toll-pathway. In response to dengue virus infection, *cactus* expression was downregulated in the mosquito, *Aedes aegypti*, whilst other components of the Toll-pathway were upregulated (Xi et al., 2008). Thus, the upregulation of *cactus* in both sexes in this study may suggest that prolonged flight led to the downregulation of the Toll-pathway to preserve energy for flight and that a time delay meant that other components of the Toll-pathway were still upregulated. Alternatively, it should be considered that flown FAW challenged with SfMNPV did upregulate *cactus* as part of their immune response, as this has been observed following immune challenge in mosquitoes (*Aedes aegypti*), flies (*Drosophila*) and in FAW following infection with the entomopathogenic nematode *Steinernema carpocapsae* (Nicolas et al., 1998; Irving et al., 2001; Sim and Dimopoulos, 2010; Huot et al., 2019).

In both sexes, *defensin* was upregulated during flight. In many insects, such as *Aedes aegypti, Apis mellifera* and *Bactrocera dorsalis*, *defensin* expression is induced by the Toll-pathway in response to infection as it is an antimicrobial peptide (Xi et al., 2008; Dong et al., 2016; Lourenço et al., 2018). *Defensin* has been shown to have an important role against fighting infection is *Spodoptera littoralis,* with a 41-fold increase observed in the defensin *Spli*Def following bacterial infection (Seufi et al., 2011). However, many *defensin* genes have been identified across the insect kingdom and without more comparative phylogenetic information, its specific function is not known. This is particularly important to consider for FAW as another *defensin*, *Spod-11-tox*, has lost its immune function (Destoumieux-Garzón et al., 2009). To address this, the potential role of *defensin* in FAW is investigated further in Chapter Five along with the other proposed components of the Toll-pathway that were identified in in this experiment.

Insect immune systems are complex, and responses can vary between species, pathogens, or the way the pathogen was administered, leading to varying results across

species in different studies (Zambon et al., 2005; Avadhanula et al., 2009; Ferreira et al., 2014). This is highlighted in Lepidoptera through numerous studies on the model organism *B. mori* so this variation is important to consider here. In one study, the JAK-STAT immune pathway was strongly upregulated in *B. mori* following viral infection with BmNPV (Liu et al., 2015), however, in a different study BmNPV did not activate the JAK-STAT immune pathway (Geng et al., 2016). Furthermore, some research on insect immune responses focusses on specific genes. For example, by measuring expression of the gene *Spätzle* to identify if the Toll-pathway is activated in response to BmNPV, however, despite upregulation of multiple components of the Toll-pathway in this study *Spätzle* was not identified as a differentially expressed gene (Liu et al., 2015). This means that it may be best to consider multiple genes within immune pathways when researching immune responses in insects.

Hdd1 and *Hdd23* were both upregulated in males and females during flight. There is considerable evidence that these genes have a role in the immune response, however it is not yet clear what this role is, or if they are involved in any of the known immune pathways. Rapid upregulation of *Hdd1* was observed following bacterial infection in the fall webworm (*Hyphantria cunea*) and silkworm (*Bombyx mori*), and similarly, *Hdd23* following viral infection with *Microplitis demolitor Bracovirus* in FAW (Woon Shin et al., 1998; Provost et al., 2011; Zhang et al., 2017). However, there is evidence of variation between species and pathogens in the expression of *Hdd23* following immune challenge as it was downregulated in response to baculovirus infections (*Spodoptera exigua nucleopolyhedrovirus* and *Autographa californica nucleopolyhedrovirus*) in *Spodoptera exigua* (Jakubowska et al., 2013).

There is much complexity behind how flight and infection change the expression of immune related genes and it is likely that the response varies between species. For instance, in the Glanville fritillary butterfly, there was no upregulation of immune related genes (*attacin* and *pelle*) during flight in individuals that were infected with bacteria whereas there were increases in infected individuals that did not fly, suggesting a trade-off between flight and immunity (Woestmann et al., 2016). However, whilst this contradicts the findings in this study, the Glanville fritillary butterfly is not a migratory species with around 80% of individuals remaining within one patch, only undertaking short, rapid flights to mate, defend territory or find food sources (Niitepõld et al., 2011). Thus, these differences in the immune response during flight observed in our study could suggest different evolutionary strategies as FAW is a migratory species that undertakes long distance flights.

Furthermore, in some species, immune-related genes may be upregulated as a stress response during flight irrespective of a pathogen being present or not. This was observed in the Glanville fritillary butterfly, in the absence of infection, there was higher expression of immune related genes during flight in females compared to males in a larger population, and in both sexes in a smaller population (Kvist et al., 2015). Additionally, in non-infected cotton bollworm (*Helicoverpa armigera*), there was an upregulation of immune related genes following long distance flight (unpublished, Appendix D, Fig. S4). Therefore, though the results of this study suggest there are differences between the sexes during flight in SfMNPV challenged FAW, it is important to consider what effect flight alone may have on immune gene expression in non-challenged individuals too. These questions are addressed in Chapter Five, which takes an in-depth look at how the Toll-pathway responds to SfMNPV challenge and flight in male and female FAW.

4.5.2 Protein production

Flight led to a strong increase in the expression of genes related to protein production in both male and female FAW. The importance of protein production during flight has strong evolutionary support, as genes associated with protein production, such as mRNA splicing, protein binding and mRNA processing, were all positively selected for in insects that had maintained their flight ability when they were compared to those that had not (Mitterboeck et al., 2017).

The similarity between the sexes suggests that protein production is likely to have a similar role in both sexes, and be essential for successful, prolonged flight. These observed increases in protein production in FAW may directly promote flight activity as has been observed in other migratory Lepidoptera. Increases in genes relating to protein production during flight in the migratory cotton bollworm were linked to the production of flight muscle proteins, and to the production of odorant binding proteins that are likely to have a role in lipid interactions that promote prolonged flight (Jones et al., 2015; Wang et al., 2020).

An increase in genes involved in the production of proteins was also identified in the non-migratory Granville fritillary butterfly 20h after flight, this was proposed to aid recovery by repairing damaged cells and replenishing energy reserves (Kvist et al., 2015). The need to repair damage caused by the stress of flight is likely to be necessary in FAW. The differences observed in the timing of the increases in protein production may suggest that evolutionarily it is more beneficial for a migratory insect to initiate these repairs during flight instead of after, as this would enable the continuation of flight over longer distances.

4.5.3 Energy metabolism, flight fuel and ROS protection

Differences in metabolism during flight between males and females have been previously observed, with peak metabolic rate negatively correlated with flight distance in males and positively correlated with flight distance in females (Niitepõld et al., 2011). Though metabolic rates were not measured in this study, the upregulation of genes associated with metabolism in females suggests that they may have had a higher metabolic rate during flight than males. Considering the high energy demands of flight, the downregulation of genes associated with metabolism observed in males was unexpected and may be due to forcing the moths to fly for five hours, as this could have led to exhaustion in some of the males that were flown.

One of the key areas of metabolic differences between males and females was in flight fuel, with males showing a preference for lipids whereas females preferred carbohydrates (Fig. 4.6). Metabolic sex differences occur in mayflies (*Siphlonurus aestivalis*) in which males have higher levels of fat reserves and use these for flight, whereas females have lower levels of fat reserves and prioritise this for reproduction so use very little fat for flight (Sartori et al., 1992). Very few studies have compared the flight fuel used by males and females in migratory Lepidoptera. However, there is some evidence that both energy sources are used by both sexes during flight, with equal amounts of lipid and carbohydrate utilised from the haemolymph in female *Anticarsia gemmatalis* during 4 hours of tethered flight (Teo et al., 1987). Alternatively, in some species (e.g. *Agrotis ipsilon*) the concentration of lipids and carbohydrates used during flight varies between whether the individual is a long- and short-distance flier (Sappington et al., 1995). Therefore, the difference in males and females in this study could be driven by behaviour as males typically fly shorter distances than females (Chapter 5, Pearson 2016).

The function of many genes is not yet clear in FAW, as much of the previous work has been carried out in other species, and genes have been identified as having different roles across the insect taxa. In this study, in males *glutathione-S-transferase* (*GST*) was upregulated during flight. Following upregulation of *GST* in the migratory cotton bollworm (*Helicoverpa armigera*) it was hypothesised that *GST* could play an important role in moths dealing with ROS produced by aerobic metabolism during flight (Jones et al., 2015). However, when *Spodoptera litura* were infected with the nematode *Heterorhabditis indica*, *GST* activity was found to increase at 6h and 9h post-infection, suggesting a potential role in detoxification following infection (Lalitha et al., 2018). Another example of an upregulated

gene without a clear function in FAW is *TGF-β1*, which was upregulated in both sexes during flight. *TGF-β1* has been identified as having a role in reducing ROS stress (Barcellos-Hoff and Dix, 1996), in the synthesis of juvenile hormone (Ishimaru et al., 2016), and in immunity by regulating the development of parasites (Vodovotz et al., 2004). Thus, further investigation into the roles of *GST* and *TGF-β1* in FAW would be necessary to increase our understanding of their differential expression during flight.

4.5.4 Reproduction

Due to the high energy demands of immunity, flight and reproduction, Bateman's principle is likely to be a key driver of differences between males and females in these three areas. In this study, we found that virus-challenged, flown males upregulated the expression of genes involved in testes development that are likely to have an important role in reproduction. In contrast, genes in females with a potential role in egg development were downregulated. This difference between males and females supports our hypothesis that the high energy demands of flight and immunity would lead to differential investment in reproduction.

This differential investment could be explained by the oogenesis-flight syndrome. This is a mechanism that has evolved in some migratory insects to enable them cope with the high energy demands of both flight and reproduction (Johnson, 1969; Rankin et al., 1994; Cheng et al., 2016b). Oogenesis-flight syndromes mean that flight is favoured in the first few days after adult eclosion and then reproductive development starts once flight has finished, usually at the cost of reduced movement (Johnson, 1969; Rankin et al., 1994; Cheng et al., 2016b). All moths in this experiment were flown on the day of emergence because the mean pre-oviposition period is 3.5 days in FAW, which would suggest that female FAW do exhibit an oogenesis-flight syndrome and favour flight over reproduction immediately following emergence (Johnson, 1987). This means that the downregulation of genes related to egg development observed here in females could be the transcriptomic effects of a mechanism such as the oogenesis-flight syndrome. However, research on invasive FAW in China found that females had strong flight ability for three days following oviposition in the lab, and up to 54% of field-trapped migrants had initiated oviposition (Ge et al., 2021). Therefore, an alternative explanation for the downregulation of genes with a role in ovarian development in challenged females is that it could be evidence of a trade-off between reproduction and immunity (i.e., lower their reproductive input to enable long distance flight to occur) that males did not face. This further highlights the evolutionary importance of differential

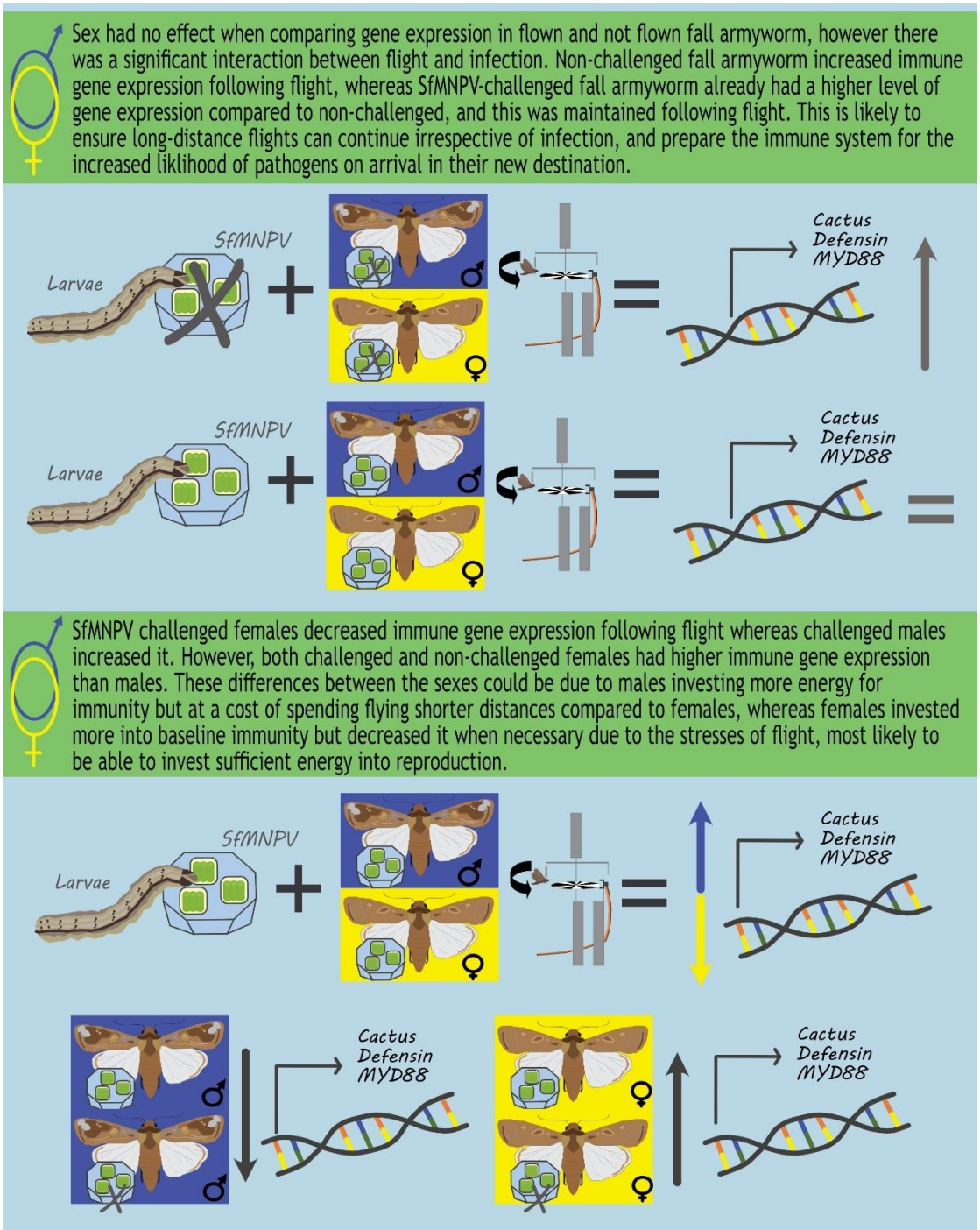
investment in reproduction and flight between male and females, and how these mechanisms are driven by Bateman's principle of males and females increasing fitness through investing differently into reproduction.

4.5.5 Conclusion

Overall these experiments showed that many genes are differentially expressed during flight in FAW, a migratory crop pest. There were many similarities between the genes differentially expressed in both sexes, including the upregulation of components of the Toll-pathway, protein production and ROS protection. The upregulation of the Toll-pathway in non-infected *H. armigera* (unpublished, Appendix D. Fig. S4) and Glanville fritillary butterfly (Woestmann et al., 2016) suggest that upregulation of these immune-related genes may be a general response to flight stress in migratory and non-migratory Lepidoptera. This makes it difficult to determine the impact that SfMNPV was having on gene expression changes during flight in this experiment, and in Chapter 5 this issue is addressed by directly comparing immune gene expression in FAW challenged or not challenged with SfMNPV.

There were some important differences highlighted in gene expression changes between males and females. This included two genes (*MYD88* and a *clip-domain serine protease*) that were significantly differentially expressed between the sexes during flight. Additionally, metabolism-associated genes were more upregulated in females, with some evidence of males utilising lipids to fuel flight whilst females use carbohydrates. Furthermore, males showed significant upregulation of some genes related to reproduction whereas females had downregulation in this area. These differences in reproductive related genes, alongside some differences in immune gene expression, support the hypothesis that Bateman's principle may help to explain the different responses to flight and infection in male and females. These differences were in key areas involved in migration, such as energy metabolism and immunity, thus are more likely to be key drivers in natural selection.

Chapter 5 - Male and female fall armyworm change Toll-pathway expression differently in response to viral challenge and flight.



5.1 Abstract

The fall armyworm (*Spodoptera frugiperda*) is native to the Americas and is a highly invasive crop pest that has spread throughout Africa, Asia and Australasia. One of the mechanisms driving this rapid spread is its ability to undergo long distance flights. Due to the presence of *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV) naturally within Africa, and the potential for it to be used as a biopesticide, this chapter aimed to determine the effects of SfMNPV on flight in males and females, and how this could change the expression of the Toll-pathway in both sexes. Following a night of flight on flight mills, RNA was extracted and RT-qPCR was used to determine gene expression changes in both males and females for four Toll-pathway genes; *cactus, defensin, MYD88* and *clip-domain serine protease*. The results showed that SfMNPV challenge and flight led to increased expression of Toll-pathway genes (*cactus, defensin* and *MYD88*) that was maintained during flight, when compared to non-challenged, not-flown FAW. However, in non-challenged FAW there was also an increase in gene expression to reach similar expression levels to those in the challenged group. These responses may be evolutionary adaptations to ensure migratory insects can successfully complete long-distance flights in the presence of covert, or novel, pathogens. Within flown FAW there were key differences in Toll-pathway response to SfMNPV between males and females. Males had lower baseline expression of Toll-pathway genes (*cactus, defensin* and *MYD88*), which increased following flight, whereas females had higher baseline expression of the genes that decreased following flight. This differential investment may be due to differing energy needs of each sex (i.e., females invest more in reproduction whereas males invest more in increasing the number of successful matings), and this was visible in the flight behaviour, with females flying longer distances than males. Overall, this study provides a novel insight into how FAW males and females change Toll-pathway expression differently in response to viral challenge (SfMNPV) and flight, and how these differences in expression are reflected in flight behaviour.

5.2 Introduction

Fall armyworm (FAW), *Spodoptera frugiperda*, is a highly invasive crop pest, native to the Americas. It spread to Africa in 2016, onto Asia in 2018, and Australia in 2020 (CABI, 2020). FAW have a very strong flight ability and undergo seasonal migrations in the Americas, with evidence of them being able to disperse up to 300 miles before oviposition (Johnson, 1987). This has enabled FAW to spread very rapidly in its newly-invaded territories, and understanding this migratory behaviour is important to better predict areas at risk.

A potential mechanism to control FAW is biocontrol using *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV) (Gómez et al., 2013; Zamora-Aviles et al., 2013). This virus can be sprayed onto infested crops and efficacies over 70% were achieved in field trials in Colombia (Gómez et al., 2013). Furthermore, it occurs naturally within FAW populations in the Americas (Shapiro et al., 1991; Niz et al., 2020; García-Banderas et al., 2020), India (Lei et al., 2020) and Africa at varying levels of prevalence depending on the location (Chapter 2). Infection can have strong impacts on migratory behaviour, potentially driving migration through 'migratory escape', triggering migration in some species, or managing populations through 'migratory culling' (Altizer et al., 2011; Hall et al., 2014; Chapman et al., 2015). However, little is known about the effects of covert SfMNPV infection on flight in FAW and this is an important area to understand due to the migratory nature of FAW that has enabled its rapid spread.

Insect immunity is complex, with a number of different mechanisms integrating to fight infection, including apoptosis, the IMD-pathway, RNAi, the JAK-STAT-pathway and the Toll-pathway (Marques and Imler, 2016). Each of these pathways is activated at different times and against different pathogens in insects, and research suggests that immune response varies between insect species and the type of pathogen (Zambon et al., 2005; Xi et al., 2008; Avadhanula et al., 2009; Cheng et al., 2016a). For example, in *Bombyx mori*, increases in immune gene expression indicated that both the IMD and Toll-pathways were induced by *Beauveria bassiana* (fungal)*, Bacillus bombyseptieus* (gram-positive) and *Escherichia coli* (gram-negative, non-pathogenic), whilst genes involved in the JAK/STAT pathway were increased in response to *Bombyx mori NPV* (BmNPV) infection (Cheng et al., 2016a). This complexity is further highlighted in *Spodoptera littoralis* that showed upregulation of Toll-pathway genes (*Toll, Phenoloxidase and Lysozyme*) following injection with dead bacteria (*Xenorhabdus nematophila*) but no change in expression following injection with live bacteria, whereas IMD-pathway genes (*Moricin* and *Relish*) were

upregulated in response to both dead and live bacteria (Cotter et al., 2019). Furthermore, in *S. littoralis* the immune response was modulated by diet availability, suggesting that the experimental conditions are important when studying insect immunity (Cotter et al., 2019).

RNA-sequence analysis showed that many immune genes were expressed differentially during flight in FAW that had been exposed to SfMNPV (Chapter 4). For some immune genes, the expression changes were consistent across both sexes, whereas for others there were significant differences between males and females (Chapter 4). These changes suggest potential sex differences in response to infection and flight at the molecular level. There is evidence of differential investment in flight leading to behavioural differences in SfMNPV challenged FAW, with males having reduced flight ability whereas females can maintain flight ability (Pearson, 2016). These differences may be due to Bateman's principle, which argues that sexual selection leads to differential investment levels between the sexes (Bateman, 1948). These differing investment levels are driven by females investing more energy into reproduction and longevity than males, whereas males face greater competition from other males (intra-masculine selection) as their reproductive success usually depends on the frequency of matings (Bateman, 1948). Consequently, females are more likely to invest resources into immunity to increase their longevity, and studies in both mammals and insects support this female-bias in immune function and male-bias in mortality following exposure to infection (Rolff, 2002; Nunn et al., 2009; Wilson and Cotter, 2013). This differential investment may have resulted in gender-specific immune strategies too, male *D. melanogaster* had higher levels of phenoloxidase following bacterial challenge (*Escherichia coli*) whereas females had higher nitric oxide levels (Moreno-García et al., 2015). Furthermore, female mealworm beetles (*Tenebrio molitor*) had higher levels of hemocytes compared to males, whereas both sexes had the same cuticle defence (first line of defence, measured by cuticle darkness) and phenoloxidase levels (second line of defence), however there was a strong negative correlation between these two defences in females suggesting a trade-off between immune traits that was not present in males (Rolff et al., 2005).

The different life history traits of males and females means that sexual selection has a greater role in males in many species, as females are often more selective about who they mate with (Andersson, 1986; McLean et al., 2012; Kelly, 2018). Sexual selection influences immunity as females are more likely to choose infection-resistant males and this resistance could be reflected in physical sexual dimorphism to ease mate choice by females; this is known as the susceptible male hypothesis (Hamilton and Zuk, 1982). Overall, there is considerable evidence that males and females face different selection pressures and that

high energy demanding activities, such as flight and immunity, are likely to be reflect these differences.

Of the immune-related genes that were differentially expressed during flight in infected FAW, four are components of the Toll-pathway (Chapter 4). This suggests that the Toll-pathway may play an important role in immunity and flight, so specific genes from this pathway were selected for further study in this Chapter. The first component was a *clip-domain serine protease* that is likely to be involved in initiating the Toll-pathway (Kanost and Jiang, 2015). It may have other roles in immunity too, serine proteases were upregulated in response to bacterial (gram-positive and gram-negative), fungal and viral challenges (Cheng et al., 2016a), and increases in serine proteases improved survival rates in *B. mori* with BmNPV infection (Nakazawa et al., 2004). The next component is *MYD88* which is a component of the Toll-induced-signalling-complex (TISC) (Valanne et al., 2011). Expression levels of *MYD88* increased following *Autographa californica NPV* (AcNPV) infection in *S. frugiperda* Sf-9 cells *in vitro* (Abe et al., 2005). Thirdly, the negative regulator of the Toll-pathway, *cactus*, was chosen. *Cactus* holds in place the transcription factor DIF so following the degradation of *cactus* the transcription factor DIF is released to trigger the production of antimicrobial peptides (Belvin and Anderson, 1995; Ferrandon et al., 2007). Though it is a negative regulator of the Toll-pathway so at higher levels it should reduce Toll-pathway activity, the upregulation of *cactus* has been observed following immune challenge in mosquitos (*Aedes aegypti*), flies (*Drosophila*) and FAW (Nicolas et al., 1998; Irving et al., 2001; Sim and Dimopoulos, 2010; Huot et al., 2019). Finally, the antimicrobial peptide *defensin* was selected as it is produced via the Toll-pathway (Xi et al., 2008; Dong et al., 2016), and strong increases in *defensin* gene expression occur following bacterial infection in *Spodoptera littoralis* (Seufi et al., 2011).

These four components (*clip-domain serine protease, MYD88, cactus* and *defensin*) are involved at different stages of the Toll-pathway. Therefore, they are good candidates to give an overview of changes in the Toll-pathway following an immune challenge and flight in a migratory insect. Gene expression changes were then quantified in response to flight and viral challenge in the two sexes. Specifically, this experiment aimed to establish:

(i) That the observed upregulation of Toll-pathway genes is linked to virus exposure and not the stress of flight alone. This is an important distinction as other work has found an upregulation of immune genes following flight in non-infected Lepidoptera (Kvist et al., 2015; Woestmann et al., 2016); and

(ii) That differences in the behavioural response to virus exposure between the sexes are further supported by different immune responses, providing additional evidence for sex-biased immunity in this species.

5.2.1 Hypotheses

This chapter tests the following hypotheses by using flight mills to quantify flight and qPCR to determine the expression of four Toll-pathway genes in FAW following one night of free-flight:

1. Due to the high energy demands of both flight and immunity, the expression of Toll-pathway genes will be different in flown and not-flown FAW, with higher expression of Toll-pathway genes expected in challenged FAW that are not-flown. This is due to the high energy demands of flight that can result in reduced immunity (Zera and Mole, 1994; Crnokrak and Roff, 2002; King, 2011).
2. Within flown FAW, Toll-pathway genes will be expressed differently between the sexes, due to differences in flight behaviour and response to SfMNPV challenge. These differences will be that:
    a. Challenged and non-challenged females will have higher immune gene expression compared to challenged and not-challenged males due to females favouring longevity
    b. Challenged flown males will have reduced immune expression compared to non-challenged males due to favouring rapid reproduction over longevity
    c. Challenged flown females will have higher immune expression compared to not-challenged females to favour longevity and fight infection
3. The changes in immunity mentioned in hypothesis 2 will lead to behavioural differences in the flight behaviours of males and females. It is predicted that females will have stronger flight ability than males in the viral-challenged group. This is due to females favouring longevity and allocating limited energy resources to flight as was observed in previous work (Pearson, 2016). This is likely to increase female fitness by improving survival rate of offspring by finding favourable habitats through long-distance flight. We hypothesise that males on the other hand will invest more in immunity to reach their destination and reproduce rapidly.

5.3 Methodology

Prior to carrying out this experiment, suitable reference genes for the experimental system of viral challenged fall armyworm and flight had to be identified. Reference genes remain stable in the experimental conditions so the genes of interest can be compared to them (Bustin et al., 2009). In non-model organisms it is particularly important to ensure that the chosen reference genes are suitable for the experimental system as the expression of potential reference genes can vary with the experimental conditions (Lu et al., 2013; Zhu et al., 2014). Once reference genes were identified, these were used to look at how gene expression changed following viral challenge and flight in both sexes, using the reference genes identified in experiment one and four selected Toll-pathway genes.

5.3.1 Identifying suitable reference genes

5.3.1.1 Culture

To ensure flight behaviour is as natural as possible it is important to use a culture recently collected from the field as this reduces the likelihood of reduced flight ability that can develop in lab strains (Davis et al., 2020; Masaki and Seno, 1990). Therefore, due to the length of time between experiments a different fall armyworm culture were used for Chapter Five than in Chapter Four. Fall armyworm larvae were collected from Bvumbwe Agricultural Research Station (Malawi) in January 2019 and fed on maize until pupation. These were then shipped to the UK and a starting population of 32 individuals (16 males, 16 females) was used for the culture. All life stages were reared in 12:12 L:D at 27°C at Lancaster University under FERA license. Once emerged, moths were mated in pairs and given 5% sugar water. Eggs were collected and placed in pots with an excess of artificial diet available (Appendix B, Table S3). First generation eggs were taken from the culture for this experiment. Whilst this culture was not screened for the presence of covert infections the culture was monitored carefully for signs of infection (e.g., stunted growth, larvae deaths leading to cadaver liquification, failure to pupate) and no larvae included in this experiment showed signs of infection. Furthermore, any effects of undetected covert infections would have been present in the non-challenged group creating the same baseline for both experimental groups.

5.3.1.2 Viral challenge

Third instar fall armyworm larvae were starved individually overnight in 96-well plates at 27°C. The following morning, in another 96-well plate, larvae were fed a diet plug with a 1µl droplet of $1x10^3$ OB/µl of *Spodoptera frugiperda multiple nucleopolyhedrovirus (*SfMNPV*)*

sourced from AgBiTech. The diet plug was 1mm diameter and was composed of the same diet the larvae were reared on. The viral dose was achieved by diluting with 20% sugar water to ensure the diet was still palatable for the larvae. Larvae were then placed back into the incubator at 27°C overnight. The following day, any larvae that did not consume the diet plug were removed from the experiment. The remaining larvae were placed into individual diet pots with an excess of artificial diet (Appendix B, Table S3). Diet pots were changed as necessary. Pupae were placed in individual pots with no diet until emergence.

5.3.1.3 Flight

All moths included in this experiment were flown on the day of emergence. Flight mills were provided by Jason Lim at Rothamsted Research (Fig 4.1, Chapter 4), and eight moths that had emerged each day from a variety of the four experimental flown groups were flown over six nights. On the day of emergence, all moths were given cotton wool soaked in 5% sugar water. Before flight, pins were attached to the thorax of the moths to allow them to be attached to the flight mills. This was carried out following standard protocols (Pearson, 2016). Moths were immobilised at 4°C for 30 minutes to one hour. Pins were then attached using EvoStick Instant Contact Adhesive® and placed back into 4°C to prevent wing damage before attaching them to the flight mills. Moths were then randomly allocated to either the not-flown or flown groups. The not-flown group was kept in a small plastic pot to prevent flight and placed in the same room as the flight mills. The flown group were attached to the flight mills and given a piece of paper to keep them stationary until lights off. At lights off, the piece of paper was removed. The moths were then left to fly overnight for 12 hours. The lights on:off cycle followed the same 12:12 L:D schedule as the incubators they had been reared in. After 12 hours, the lights were turned on and moths were placed in 2ml Eppendorf tubes and immediately frozen in liquid nitrogen. Moths were then stored at -80°C until RNA extractions took place. The flight room was maintained between 25-28°C.

5.3.1.4 RNA extraction

Six samples were extracted from each group, apart from the challenged flown male and challenged flown female groups where five were selected. This difference in sample size was due to methodological constraints of sample numbers for gene amplification as all samples needed to fit on one 96-well qPCR plate. Samples were randomly selected from each flight mill experiment for RNA extraction. RNA was extracted from the heads of moths using the New England Biolabs Monarch® Total RNA Miniprep Kit following the standard protocol for 30-50mg tissue. The head was selected as it is a key component of the central nervous

system and plays an important role in insect behaviour (Hai-Xu et al., 2017). Following RNA extraction samples were stored at -80°C.

5.3.1.5 Reverse transcription

Reverse transcription was carried out using the Primer Design Precision nanoScript™ 2 Reverse Transcription Kit. RNA at a concentration of 0.14 to 1.84µg was used for reverse transcription. The cDNA product was then diluted 1:10 for use in the qPCR experiments.

5.3.1.6 Gene amplification

Six potential reference genes were designed by Primer Design GeNorm kit for amplification to test their stability in *S. frugiperda* (Table 5.1). Each reaction contained 1µl 300nM primer solution, New England Biolabs Luna® Universal qPCR Master Mix, 4µl ddH_2O and 5µl of the 1:10 diluted cDNA product. An ABI 7500 fast qPCR machine was used for amplification using the SYBR settings with a standard melt curve. The amplification conditions were an initial denaturation step of 95°C for 1 minute, 40 cycles of 95°C for denaturation and a 60°C extension step.

Table 5.1 Primer details for the potential reference genes.

Gene name	Accession number	Anchor nucleotide
Arginine kinase (AK)	KC262639.1	723.5
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	KC262638.1	319.5
Elongation factor 1-alpha (EF1)	U20139.1	156
Voucher SPO-LSU21C 28S ribosomal RNA gene (28S)	HQ178622.1	267
Spodoptera litura myosin light chain alkali LOC111356083 (Myosin)	XM_022970318	107
Ribosomal protein L35A (L35)	AF400197.1	343

5.3.1.7 Data analysis to identify suitable reference genes

The software qbase+ was used for identifying suitable reference genes using the GeNorm analysis option (Vandesompele et al., 2002; qbase+, 2017). GeNorm calculates the gene-stability measure (GeNorm M) using the arithmetic mean of the pairwise variations of the genes (Vandesompele et al., 2002). The genes are then ranked using stepwise exclusion from the least to most stable. The GeNorm V method is also used to determine the number of reference genes that should be used. This uses pairwise variation ($V_{n/n+1}$) of normalization factors (geometric mean), and genes are added until V is optimal at <0.15 (Vandesompele et al., 2002). Further analysis was carried out to calculate the mean CT and standard deviation for each experimental group. Finally, the mean CT per sample in relation to the concentration of RNA that went into the reverse transcription was considered to ensure the

high variation in starting RNA concentration was not influencing selection, thus correcting for not stabilising the RNA concentration across samples prior to reverse transcription.

5.3.1.8 Two reference genes were selected for this experimental system.

When doing qPCR work in a novel experimental system it is important to ensure that the reference genes used are stably expressed across treatment groups. The conservative GeNorm M method was used to identify the most stable genes based on the arithmetic mean (Vandesompele et al., 2002; qbase+, 2017). This method highlighted L35 as the most stable reference gene across all samples, and EF1 as the second most stably expressed gene (Appendix E, Fig. S5). GeNorm V analysis determined the number of reference genes that should be used based on pairwise variation ($V_{n/n+1}$) of normalization factors, with genes being added in order of stability until V is <0.15, however, in this experiment this meant that all six potential reference genes were recommended. This was not feasible due to the costs and time involved to normalise to several target genes. GeNorm analysis is designed for fewer experimental groups and over eight potential reference genes, however we only had six in a non-model organism, so it was decided to investigate the reference genes further. Looking at the different experimental groups the most stable genes were EF1 and L35, as these had the lowest standard deviation ranges across all groups (Table 5.2, Fig. 5.1). Considering this, EF1 and L35 were chosen as reference genes. For this type of research two reference genes is in line with what similar studies have used, with many studies only using one (Cankar et al., 2006; Seufi et al., 2011; Barthel et al., 2015).

Table 5.2 Average and standard deviation Ct values of the potential reference genes.

Experimental Group	Ct Mean	28S	AK	EF1	GAPDH	L35	Myosin
Flown Control Female	Average	18.51	26.38	23.11	29.46	23.41	25.12
	StdDev	3.05	2.96	1.63	2.94	1.84	3.22
Flown Control Male	Average	15.14	22.48	23.19	26.85	22.99	22.44
	StdDev	2.36	3.46	1.72	3.56	1.07	2.92
Flown Challenged Female	Average	17.91	25.58	23.29	29.85	23.94	24.70
	StdDev	1.71	3.64	1.24	3.80	2.99	2.70
Flown Challenged Male	Average	18.11	24.88	24.37	28.47	24.16	24.43
	StdDev	2.42	3.02	1.07	1.94	1.37	1.77
Not-flown Control Female	Average	19.21	27.14	23.43	28.56	24.64	25.62
	StdDev	2.00	3.36	1.12	2.56	3.14	2.08
Not-flown Control Male	Average	18.59	26.10	23.86	28.25	24.67	23.70
	StdDev	2.18	4.86	1.34	2.52	3.24	2.57
Not-flown Challenged Female	Average	19.27	26.35	23.49	28.63	23.93	25.56
	StdDev	1.17	3.50	1.37	1.84	2.44	2.21
Not-flown Challenged Male	Average	17.63	24.10	23.92	27.25	24.27	23.83
	StdDev	2.75	2.70	0.86	1.71	1.87	1.60

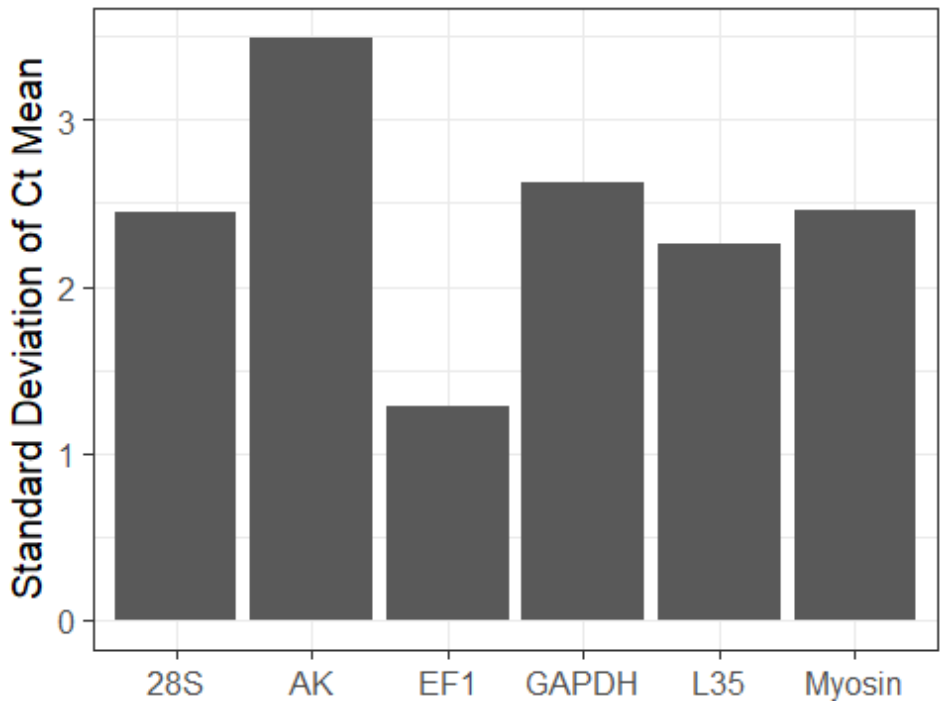


Figure 5.1 Standard deviation of reference genes. The lowest standard deviation reflects the most stable reference gene, which was EF1 and L35.

5.3.2 Quantifying Toll-pathway gene expression

5.3.2.1 Culture, viral challenge, and flight

The methods for rearing, viral challenge and flight mills were the same as in experiment one with the only alteration that the eggs taken were from the second generation of FAW.

5.3.2.2 RNA extraction

Moths in the flown group that had not-flown at least 1000m during the 12 hours on the flight mill were excluded from analysis. Samples were selected from the flight mill experiment for RNA extraction and future RNA steps. Six samples were selected from each treatment group, apart from the not-flown challenged female group from which 5 were selected. RNA was extracted from the heads of moths using the Qiagen AllPrep DNA/RNA Mini Kit following the standard protocol for 30mg tissue. The head was selected as it is a key component of the central nervous system and plays an important role in insect behaviour (Hai-Xu et al., 2017). Following RNA extraction samples were stored at -80°C.

5.3.2.3 Reverse transcription

Reverse transcription was carried out using the standard protocol of the New England Bioloabs LunaScript™ RT SuperMix Kit. RNA at a contraction of 0.2µg was used for reverse transcription. The cDNA product was then diluted 1:10 for use in the qPCR experiments.

5.3.2.4 Gene amplification

Four genes were selected based on the RNA-sequence results (Chapter 4) and primers were designed for amplification in *S. frugiperda* by Primer Design (Table 5.3). Each reaction contained 1µl 300nM primer solution, 10µl New England Biolabs Luna® Universal qPCR Master Mix, 4µl ddH_2O and 5µl of the 1:10 diluted cDNA product. An ABI 7500 fast qPCR machine was used with SYBR settings and a standard melt curve. The amplification conditions comprised of an initial denaturation step of 95°C for 1 minute, 40 cycles of 95°C for denaturation and a 60°C extension step.

Table 5.3 Primer information for the four Toll-pathway genes and the reference genes.

Gene name	Accession number	Anchor nucleotide
clip-domain serine protease: GSSPFG00020560001.3	Not available	96
Cactus: GSSPFG00001145001.2	Not available	497
Defensin: GSSPFG00008360001.3	Not available	67
MYD88: GSSPFG00016587001.3	JQ687409	1014
Reference: Elongation factor 1-alpha (EF1)	U20139.1	156
Reference: Ribosomal protein L35A mRNA (L35)	AF400197.1	343

5.3.2.5 Gene expression analysis

Relative CT expression values were calculated using the DeltaCT method (Winterhalter and Fedorka, 2009; Costa et al., 2013). Samples with only one reference gene amplified were removed from analysis (N = 3). This is calculated by the formula:

DeltaCT = Average(CT Reference Genes) – CT Target Gene

This normalised the data for each gene of interest and considered inter-plate variation. All statistical analysis was carried out in R (v 3.6.1). A multivariate analysis of variance (manova) was used to test the hypothesis that all four candidate genes are affected by flight, sex or treatment group, as well as considering the effects of each of the genes individually. Model simplification removed non-significant variables from the model, and an anova compared models to confirm there was no significant effect of removing these variables. A principal component analysis (PCA) was used to investigate flight in more detail. Principal components (PC) were then extracted and used in a manova to look at gene expression in flown males and females across treatment groups.

5.4.2 Results

5.4.2.1 Hypothesis 1: Compared to those not-flown, Toll-pathway expression was increased in non-challenged flown FAW but maintained at a similar level in challenged flown FAW.

A multivariate analysis of variance (manova) was used to test the hypothesis that the interaction between flight, sex or treatment group changed gene expression in all four candidate genes, as well as considering the effects of each gene individually. A manova was chosen as it allowed the expression of all four genes (as the dependent variables) to be compared simultaneously instead of each gene individually, as would be the case with the application of anova.

In the final model there was a significant interaction between flight and treatment group (viral-challenged or not) across the four Toll-pathway genes (V=0.14, $F_{1,78}$ =3.43, *P*=0.0012). This suggests that there is a complex interaction between the response of each gene to flight and virus exposure. Model simplification indicated that sex did not explain a significant amount of variation and it was removed from the model, and there was no significant difference between the full and simplified model (V=0.02, $F_{1,78}$ =0.44, *P*=0.777).

Due to the high levels of significance, the effects of each of the four genes were studied individually from the manova output (Fig. 5.2, Table 5.4).

Cactus is a negative regulator in the Toll-pathway, with its degradation triggering the release of the transcription factor DIF from the Toll-induced-signalling-complex (TISC) and consequently the transcription of antimicrobial peptides (Valanne et al., 2011). In this experiment, there was a significant interaction between flight activity and viral infection ($F_{1,78}$=4.12, *P*=0.046). The expression of *cactus* was increased in non-challenged flown moths compared to those that were not-flown, whereas *cactus* expression was maintained at the same level in both flown and not-flown challenged moths (Fig. 5.2).

Defensin is an antimicrobial peptide that is produced following activation of the Toll-pathway (Xi et al., 2008). There was a significant interaction between flight activity and treatment group for *defensin* ($F_{1, 78}$=5.61, *P*=0.020), with on average an increase of expression in the non-challenged flown group whereas expression was maintained at the same level in both flown and not-flown challenged moths (Fig. 5.2). However, there was more variation in the expression of defensin in flown, non-challenged FAW meaning some individuals did not show an increase in expression compared to the not-flown challenged group.

MYD88 is involved in the Toll-pathway as part of the TISC (Valanne et al., 2011). There was a significant interaction between treatment group and flight in the expression of *MYD88* ($F_{1, 78}$=7.10, *P*=0.009). In the non-challenged group expression of *MYD88* was on average higher in flown moths compared to those not-flown, whereas expression in the challenged group was slightly reduced on average in response to flight (Fig. 5.2).

In the Toll-pathway, *clip-domain serine protease* genes are thought to be involved in the initiation of the signalling cascade that leads to the formation of TISC and the transcription of antimicrobial peptides (Valanne et al., 2011; Kanost and Jiang, 2015). There was no significant interaction between expression and infection status or flight ($F_{1,78}$=0.94, *P*=0.334). Expression of *clip-domain serine protease* was higher in the non-challenged group compared to the challenged group ($F_{1,78}$=8.48, *P*=0.005), and flight did not have a significant effect ($F_{1,78}$=0.05, *P*=0.832).

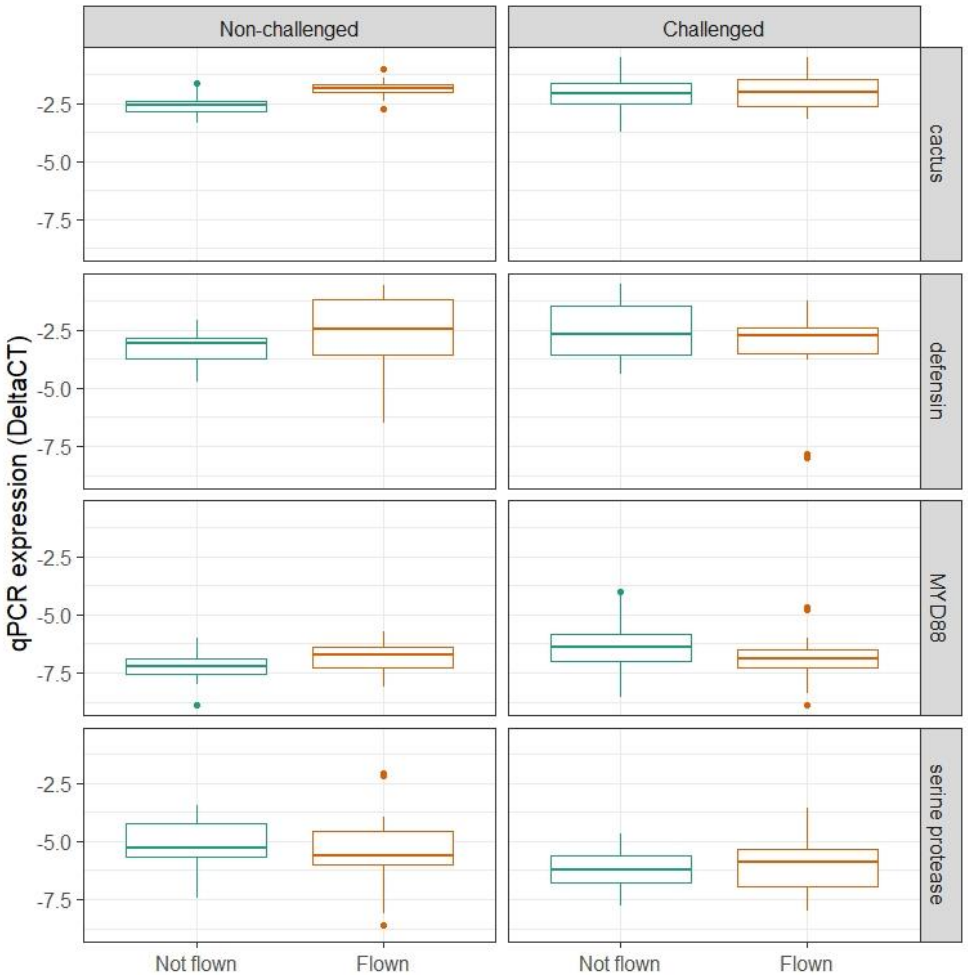


Figure 5.2 Box plots comparing the expression (Delta CT) of *cactus*, *defensin*, *MYD88* and *clip-domain serine protease* in challenged and non-challenged FAW that were either flown or not-flown. The box plots show the variation in expression changes in fall armyworm, with the mean expression change shown by the central line. This reveals that expression changes triggered by flight were more variable in the non-challenged group, with flight leading to increases in cactus, defensin and MYD88.

Table 5.4: **The final manova model output for individual genes when comparing flown to not-flown FAW.** Significant *P* values (*P*>0.05) are shown in bold.

	Df	Mean sq	F value	*P value*
Cactus				
Flight activity (flown or not-flown)	1	2.75	6.16	**0.015**
Treatment group (challenged, non-challenged)	1	0.80	1.78	0.186
Flight activity: Treatment group	1	1.84	4.12	**0.046**
Defensin				
Flight activity (flown or not-flown)	1	0.10	0.05	0.825
Treatment group (challenged, non-challenged)	1	0.03	0.01	0.909
Flight activity: Treatment group	1	11.76	5.61	**0.020**
MYD88				
Flight activity (flown or not-flown)	1	0.05	0.05	0.829
Treatment group (challenged, non-challenged)	1	3.08	3.19	0.078
Flight activity: Treatment group	1	6.86	7.10	**0.009**
clip-domain serine protease				
Flight activity (flown or not-flown)	1	0.07	0.05	0.832
Treatment group (challenged, non-challenged)	1	13.70	8.48	**0.005**
Flight activity: Treatment group	1	1.53	0.94	0.334

5.4.2.2 Hypothesis 2: In flown fall armyworm, gene expression was significantly affected by a three-way interaction between flight behaviour, sex and SfMNPV challenge.

Overall, 16 flight variables were calculated from the flight mill data (Table 5.5). Many of these variables are highly correlated, therefore a principal components analysis (PCA) was carried out to enable all components of flight behaviour to be considered when looking at gene expression (Table 5.5, Fig. 5.3). This grouped the flight mill data into three clusters; distance / duration of flight, speed of flight and number of flights (Table 5.5, Fig. 5.3).

The PCA shows less variation in the distribution of both challenged and non-challenged females compared to males. This suggests that the flight activity of males is more variable irrespective of viral challenge (Fig. 5.3). Variation in the flight of non-challenged males and females is explained by variables associated with flight speed (Fig. 5.3). On the other hand, variation in challenged males during flight was linked to variables associated with distance flown (Fig. 5.3). Flight activity in challenged females differed compared to the other three groups, with the number of flights potentially influencing this (Fig. 5.3).

The most important principal components (PC) were PC1, PC2, PC3 and PC4, as these all had an eigenvalue greater than 1, meaning these each accounted for more variation than the original variables. These four PC combined account for 87% of the variation in the data, and

each PC largely accounted for specific behavioural aspects, such as distance (PC1), speed (PC2), duration (PC3) and number of flights (PC4) (Table 5.5). Thus, by using the four principal components in a manova to analyse gene expression most of the variation in flight behaviour was included. To ensure that all four PCs were needed in the model, a manova was carried out using PC1, PC1+PC2 and PC1+PC2+PC3 (model details in Appendix E, Table S4). The full model with PC1+PC2+PC3+PC4 explained the most variation in the data, as the AIC was lowest for this model (AIC PC1:3= 65, AIC PC1:4= 25). Furthermore, an anova comparing the models showed that the model containing PC1+PC2+PC3+PC4 was significantly different to all other models, suggesting that the variation explained by PC1+PC2+PC3+PC4 had a significant role in explaining the variation of gene expression (*P*<0.0001 in each comparison, model details in Appendix E, Table S5).

In flown moths, there was a significant three-way interaction related to gene expression between flight behaviour, viral challenge, and sex (MANOVA: V=1.36, $F_{2,22}$ =10.67, *P*=<0.0001). This shows that gene expression is highly variable in FAW and is influenced by both viral challenge and flight, and that this variation is different in males and females (Fig. 5.3). Considering this three-way interaction, each gene was considered individually from the manova output (Table 5.6).

There was a significant effect of flight behaviour on *cactus* expression ($F_{4,22}$ =6.53, *P*=0.001, Table 5.6). In the PCA, *cactus* was positively correlated to distance-related variables (Fig. 5.3). *Cactus* expression is slightly lower in non-challenged males compared to females, whereas in the challenged group females had lower expression than males (Fig. 5.4). This is due to challenged females decreasing *cactus* expression whereas challenged males increased expression of *cactus,* however this relationship was marginally non-significant ($F_{4,22}$ =2.51, *P*=0.071).

There was a significant three-way interaction between flight, viral challenge, and sex in *defensin* expression ($F_{2,22}$=23.68, *P*=<0.0001, Table 5.6). *Defensin* was positioned centrally in the PCA biplot, suggesting that one area of flight behaviour alone did not strongly influence expression (Fig. 5.3). *Defensin* expression in challenged and non-challenged males was very similar, whereas expression was higher in non-challenged females compared to challenged females (Fig. 5.4). In both non-challenged and challenged groups, expression was higher in females than males (Fig. 5.4).

There was a significant interaction between flight behaviour and sex in *MYD88* expression ($F_{4,22}$ =3.26, *P*=0.030, Table 5.6). As with *cactus*, in the PCA *MYD88* was positively correlated

to distance related variables (Fig. 5.3). *MYD88* expression is higher in non-challenged females compared to males (Fig. 5.4). However, in the challenged group *MYD88* expression is very similar in both sexes, with a decrease in female expression and an increase in male expression when compared to the non-challenged group (Fig. 5.4).

Clip-domain serine protease had a significant three-way interaction in this model ($F_{2,22}$ =5.30, *P*=0.013, Table 5.6). C*lip-domain serine protease* expression fitted into the PCA closest to the challenged group and was positively correlated with the variable representing the number of flights (Fig. 5.3). Expression was slightly higher in females compared to males in both treatment groups, and for both sexes' expression was lower in the challenged group compared to the non-challenged group (Fig. 5.4).

Table 5.5 The vector loadings of variables in the PCA, and the percentage of variation explained in each of the principal components (PC) produced. Variable loadings with a high contribution (classed as >0.3 or <-0.3) are shown in bold. Only PC with an eigenvalue >1 are shown here, a full table with all 16 PC is in Appendix E, Table S4.

Variable		PC1	PC2	PC3	PC4
Distance	Total distance	**0.33**	-0.04	0.03	-0.08
	Average flight distance	0.29	-0.05	**-0.38**	0.19
	First flight distance	0.19	-0.23	**0.48**	0.24
	Furthest flight distance	**0.32**	-0.22	-0.08	0.01
	Longest flight distance	**0.32**	-0.22	-0.08	0.01
Duration	Total duration	0.28	-0.22	0.02	**-0.33**
	Average flight duration	0.29	-0.06	**-0.41**	0.15
	First flight duration	0.17	-0.27	**0.50**	0.21
	Longest flight duration	0.29	-0.26	-0.11	-0.13
Speed	Max speed	0.24	**0.34**	0.09	-0.26
	Average flight speed	0.20	**0.30**	-0.14	**0.34**
	First flight mean speed	0.17	**0.34**	0.04	**-0.40**
	First flight max speed	0.24	**0.30**	0.13	**-0.30**
	Longest flight speed	0.15	**0.32**	0.28	0.27
	Longest flight max speed	0.25	0.21	0.20	0.02
Number	Number of flights	-0.12	**-0.32**	0.09	**-0.45**
Eigenvalue		7.67	3.18	1.78	1.29
Standard deviation		2.77	1.78	1.33	1.13
Variance explained (%)		0.48	0.20	0.11	0.08
Cumulative variation explained (%)		0.48	0.68	0.79	0.87

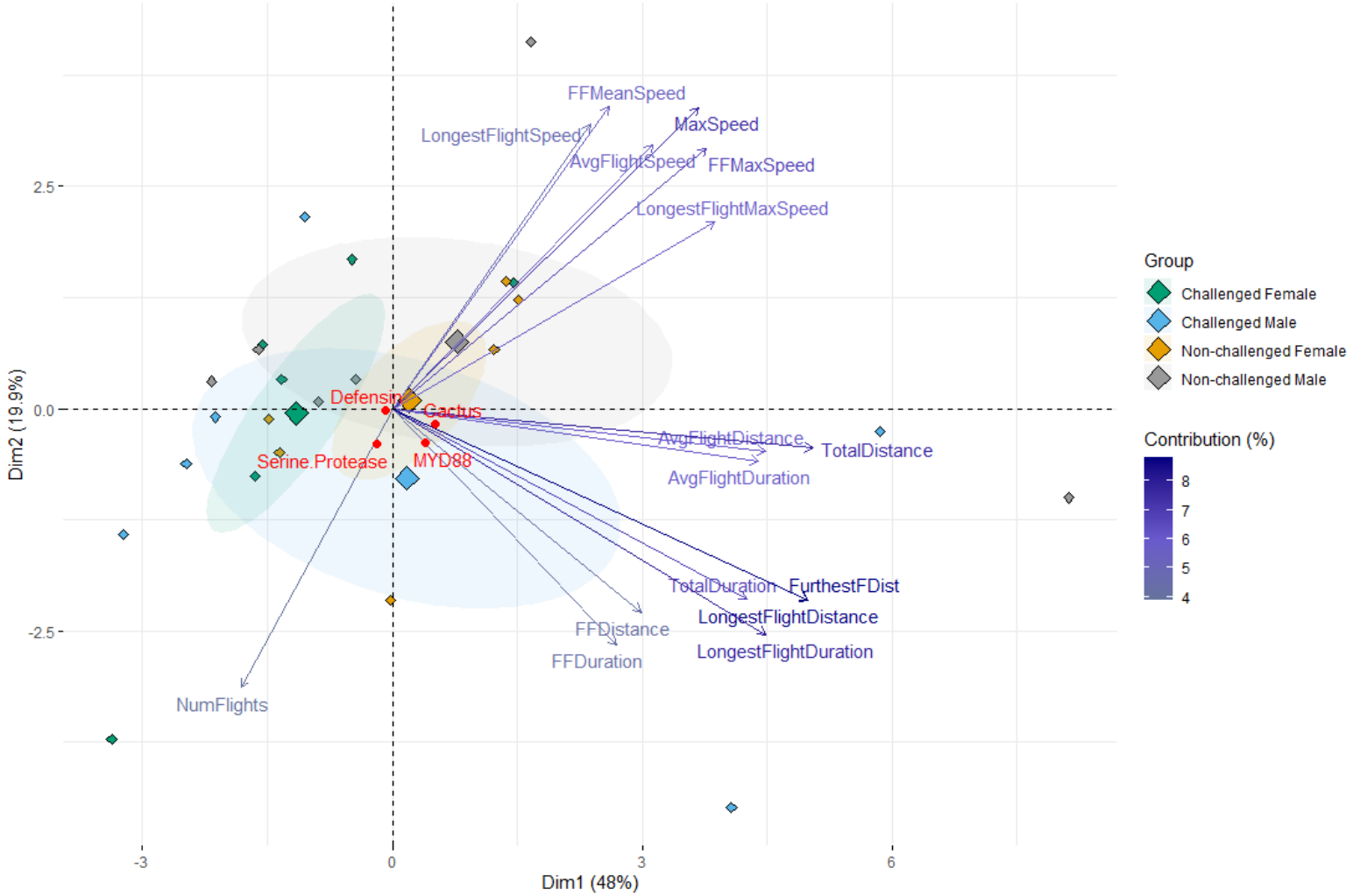


Figure 5.3: **Principal components (PC) analysis biplot for flight and Toll-pathway gene expression.** This shows the first two PC plotted against each other, and how challenged and non-challenged males and females fit into this. The colour shows the contribution (%) of each variable to the principal components, whilst the direction shows variables correlated with each other. Smaller diamonds represent individuals and larger diamonds show the mean of each Group. The ellipses around each group mean point show the 95% confidence level. The expression of the four candidate genes was predicted and the position of each gene on the biplot is shown by a red circle and corresponding red label.

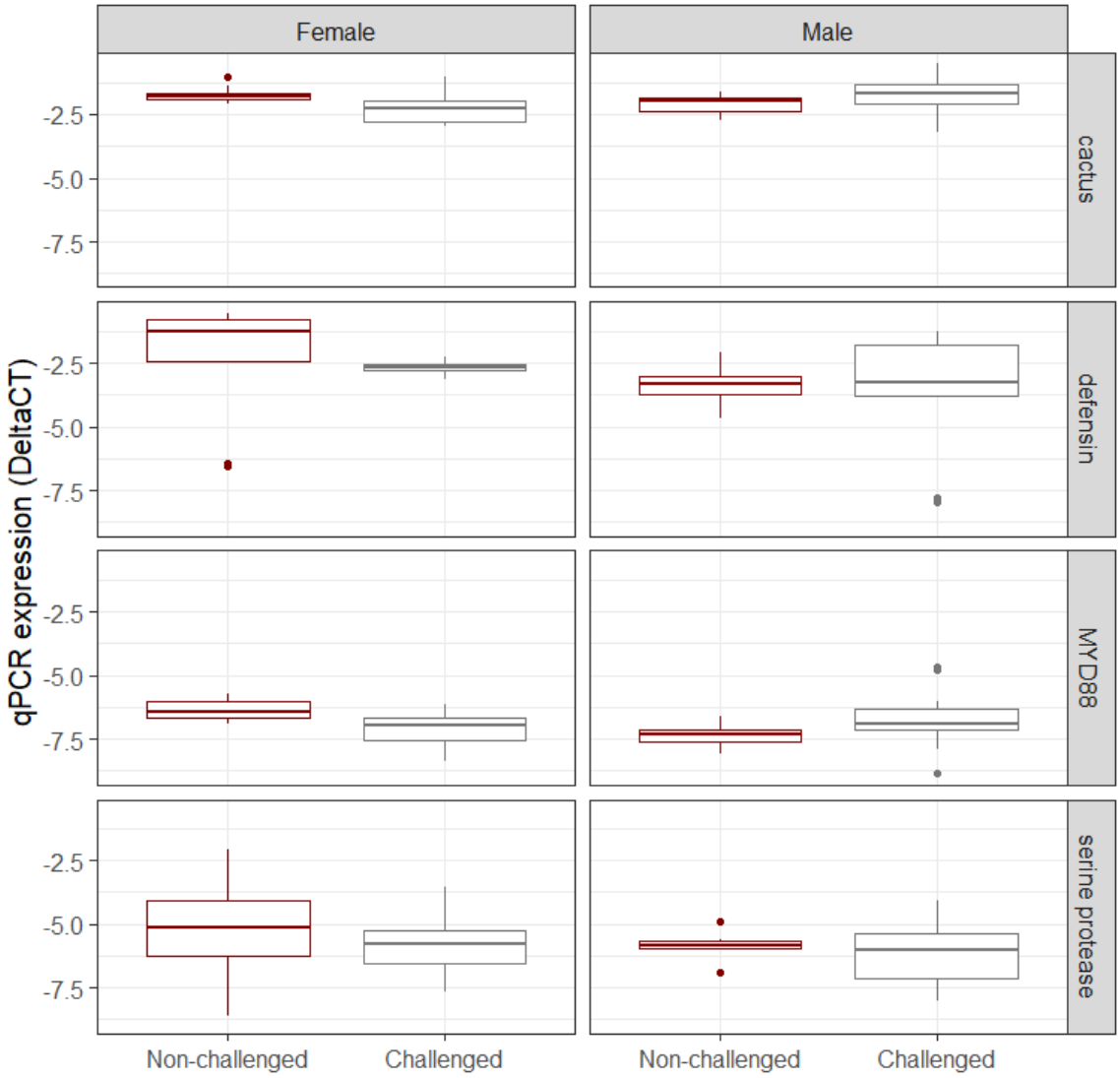


Figure 5.4 Box plots comparing the expression (Delta CT) of *cactus*, *defensin*, *MYD88* and *clip-domain serine protease* in challenged and non-challenged FAW that were flown. The box plots show the variation in expression changes in fall armyworm, with the mean expression change shown by the central line. This shows that on average, flown challenged females reduced expression of cactus, defensin and MYD88 whereas flown challenged males maintained, or slightly increased, expression.

Table 5.6: **Manova results for *cactus*, *defensin*, *MYD88* and *clip-domain serine protease* when looking at flown moths only.** Significant (<0.05) *P* values are in bold.

	Df	Mean sq	F value	*P* value
Cactus				
PC 1 to 4 (flight behaviour)	4	1.53	6.53	**0.001**
Treatment group (challenged, non-challenged)	1	0.00	0.01	0.922
Sex	1	0.45	1.94	0.178
PC: Treatment group	4	0.12	0.53	0.715
PC: Sex	4	0.59	2.51	0.071
Treatment group: Sex	1	0.37	1.60	0.220
PC: Treatment group: Sex	2	0.34	1.45	0.255
Defensin				
PC 1 to 4 (flight behaviour)	4	17.35	182.87	**<0.001**
Treatment group (challenged, non-challenged)	1	1.83	19.26	**<0.001**
Sex	1	0.02	0.22	0.641
PC: Treatment group	4	9.76	102.88	**<0.001**
PC: Sex	4	0.94	9.89	**<0.001**
Treatment group: Sex	1	0.73	7.67	0.011
PC: Treatment group: Sex	2	2.25	23.68	**<0.001**
MYD88				
PC 1 to 4 (flight behaviour)	4	3.30	10.62	**<0.001**
Treatment group (challenged, non-challenged)	1	0.31	1.00	0.327
Sex	1	0.06	0.20	0.661
PC: Treatment group	4	0.63	2.02	0.127
PC: Sex	4	1.01	3.26	**0.030**
Treatment group: Sex	1	1.02	3.27	0.084
PC: Treatment group: Sex	2	0.19	0.61	0.553
Clip-domain Serine Protease				
PC 1 to 4 (flight behaviour)	4	8.65	9.90	**<0.001**
Treatment group (challenged, non-challenged)	1	3.47	3.97	0.059
Sex	1	0.51	0.59	0.452
PC: Treatment group	4	3.89	4.45	**0.009**
PC: Sex	4	0.80	0.92	0.472
Treatment group: Sex	1	1.73	1.97	0.174
PC: Treatment group: Sex	2	4.64	5.30	**0.013**

5.4.2.3 Hypothesis 3: Flight behaviour was different in male and female fall armyworm.

The four key PCs largely accounted for specific flight behavioural aspects such as distance (PC1), speed (PC2), duration (PC3) and number of flights (PC4) (Table 5.5). A variable representing each of these four aspects of flight were plotted on boxplots to give an indication of the behaviour in the challenged and non-challenged males and females (Fig. 5.5). To avoid over-analysing the data, no statistical tests were done to directly compare males and females based on individual flight variables, but the PCA found a significant interaction between flight behaviour, viral challenge, and sex ($V=1.36$, $F_{2,22}=10.61$, $P<0.0001$). There was very little difference between the four groups considering the number of flights (Fig. 5.5A) and mean speed flown (Fig. 5.5B). On average, viral challenged males and females flew shorter distances and for less time than non-challenged males and females (Fig 5.5C and Fig. 5.5D). However, on average both challenged and non-challenged females flew further than non-challenged males, though there was more variation in the response of males with some individuals flying further and longer than females (Fig 5.5C and Fig. 5.5D).

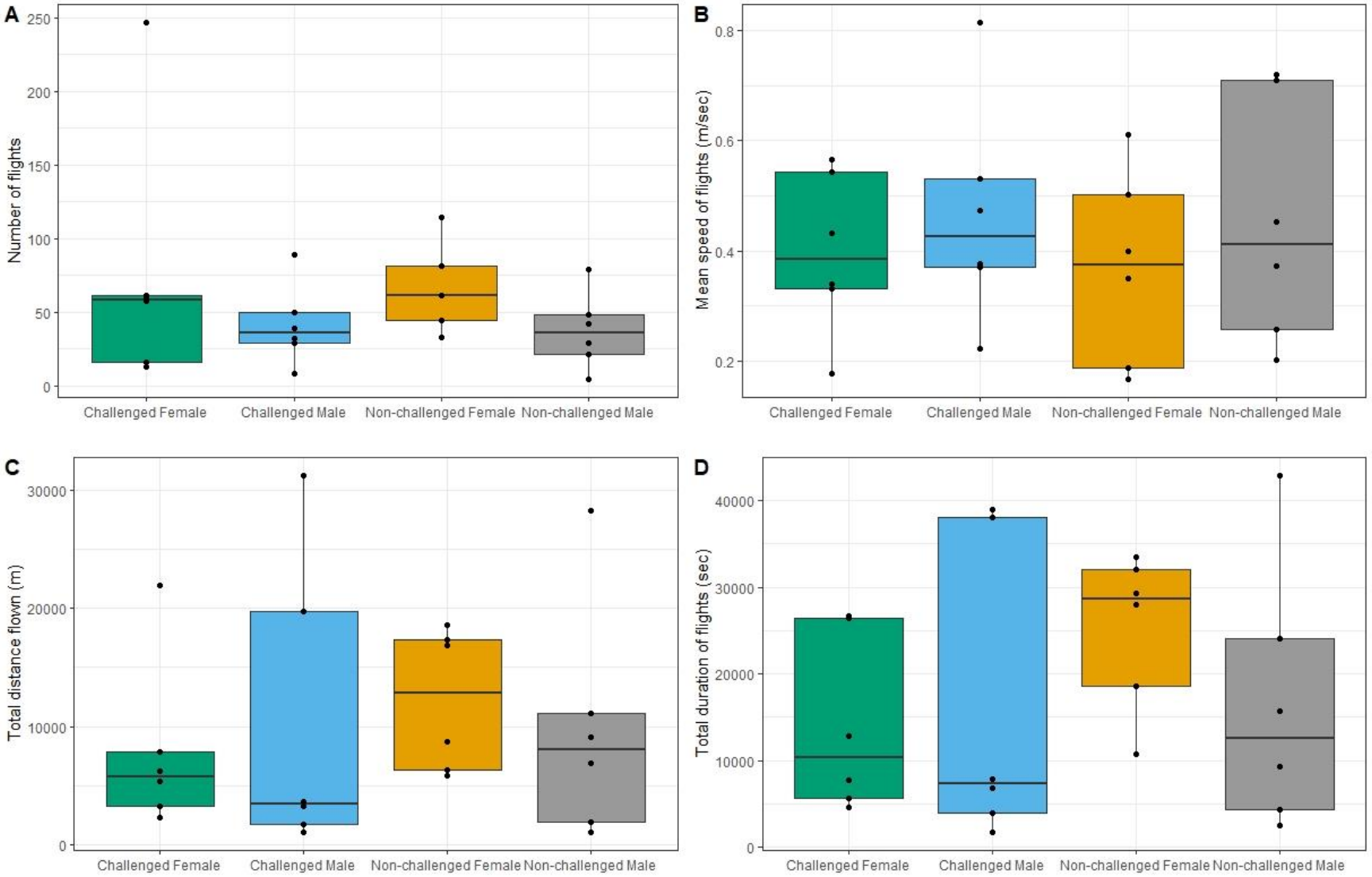


Figure 5.5 Boxplots of the flight behaviours. Four key areas of flight behaviour were detected by the PCA, one variable representing each group was selected to compare the experimental groups, A) number of flights, B) mean speed (m/sec), C) total distance (m), D) total duration (sec) in flown FAW.

5.5 Discussion

Overall, these results suggest that FAW change Toll-pathway gene expression following immune challenge with SfMNPV and flight. However, sex-specific differences were only apparent when comparing flown moths, but not when comparing flown and not-flown moths. Combined, these results show that the Toll-pathway may play an important role in the response to SfMNPV challenge, and that this response is different in males and females, most likely due to different life-history strategies.

5.5.1 Following SfMNPV challenge Toll-pathway gene expression is increased to a similar level in flown and not-flown fall armyworm compared to non-challenged not-flown fall armyworm.

Insect immunity is highly complex with different immune challenges initiating different immune responses (Cheng et al., 2016a). This study builds on the current knowledge of insect immunity by showing that the Toll-pathway immune gene expression plays an important role in the response to SfMNPV in FAW. The lowest expression of three of the genes studied (*cactus, defensin, MYD88*) was in those fall armyworm that were non-challenged and not-flown, suggesting that without the stress of flight and the presence of SfMNPV these genes were expressed at relatively low levels. Higher levels of expression occurred in the non-challenged flown fall armyworm for three genes (*cactus, defensin* and *MYD88*). In both the not-flown and flown challenged fall armyworm expression levels of *cactus, defensin* and *MYD88* were very similar to those in the non-challenged flown group, and expression was maintained at the same level for *cactus* and *defensin* in both the not-flown and flown groups. This maintained upregulation observed in challenged FAW suggests that the Toll-pathway is probably important in response to SfMNPV. Furthermore, the previous chapter (Chapter 4) found no evidence of other immune pathways, such as the JAK/STAT-pathway or IMD-pathway, responding to viral-challenge in flown or not-flown FAW, further supporting the potential importance of the Toll-pathway response.

In the non-challenged flown fall armyworm there was an increase in expression of *cactus, defensin* and *MYD88* compared to the not-flown group. This contradicts Hypothesis 1 which states that Toll-pathway genes would be higher in challenged FAW that were not-flown compared to those that were, due to the high energy demands of flight. Maintaining an immune response could help fight the viral infection and increase survival time, giving FAW time to reach their destination and reproduce. Being able to maintain an immune response may be an adaptation of migratory Lepidoptera, enabling them to undergo long-

distance migratory flights even when faced with an immune challenge. In the field, covert infections of *Spodoptera exempta nucleopolyhedrovirus* were maintained in migratory populations of the African armyworm (*Spodoptera exempta*), suggesting that they had managed to complete long-distance flights with covert viral infections (Vilaplana et al., 2010). Similarly, immunity was unaffected by flight in the Monarch butterfly (*Danaus plexippus*), with no evidence of a shift in phenoloxidase and lysozyme activity levels caused by short flights over a period of a few days (Fritzsche McKay et al., 2016). Combined, these studies, alongside the findings presented here, suggest that migratory Lepidoptera may have adapted to maintain high immunity levels to respond to immune challenges despite the high energy demands of flight.

There was an increase of Toll-pathway gene expression in non-challenged flown FAW. This was unexpected as there was no pathogen present to trigger an immune response, and previous work in migratory sand crickets (*Gryllus firmus*) showed that migratory behaviour leads to reduced immunity as trade-offs occur to overcome the high energy demands of both immunity and flight (Zera and Mole, 1994; Crnokrak and Roff, 2002; King, 2011). The increase in immune gene expression in flown FAW despite no viral challenge is hypothesised to be because of two reasons. Firstly, in the non-migratory Glanville fritillary butterfly (*Melitaea cinxia*), immune-related gene expression was increased in non-infected individuals following short bursts of flight, most likely as part of a stress response (Kvist et al., 2015). Therefore, the observed increases in *cactus*, *defensin* and *MYD88* in the non-challenged group could be triggered by the stress of flight. Alternatively, research has shown that migratory species are exposed to higher levels of pathogens during migration and more novel pathogens in the destinations they reach (Fritzsche McKay and Hoye, 2016; Altizer et al., 2011; Koprivnikar and Leung, 2015; Chapman et al., 2015). As an evolutionary response to this increased exposure some migratory species have increased immunity compared to non-migratory species following flight, such as larger immune defence organs (i.e., bursa, spleen) in birds (Møller and Erritzøe, 1998), rapid restoration of immune function in birds at stopover sites (Eikenaar et al., 2020), and higher baseline and functional immunity during migration in Nathusius' pipistrelles (*Pipistrellus nathusii*) (Voigt et al., 2020). Therefore, the observed increase in Toll-pathway gene expression in non-challenged FAW during flight may be a migratory adaptation triggered by long-distance flight to prepare for the increased likelihood of being exposed to disease. Further supporting this notion, an upregulation of immune-related genes was observed in non-challenged migratory cotton bollworm (*Helicoverpa armigera*) that were flown overnight (Chapter 4, Appendix D, Fig. S4).

Additionally, the concentration of haemocyte (immune cells that function in the primary line of defence) was positively correlated with distance flown in Monarch butterflies, showing that as distance increased, so did cellular immunity (Fritzsche McKay et al., 2016).

To date, insufficient work has been carried out to determine if either of these hypotheses can be applied to migratory insects. These results highlight that improving understanding of the effects of long- and short-distance flights on immune function is an important area for future research.

Overall, *defensin*, *cactus* and *MYD88* followed a very similar pattern of expression across treatment groups when comparing flown and not-flown moths. This similarity in expression would be expected as they are all components (*cactus* and *MYDY88*) or products (*defensin*) of the Toll-pathway. As the *clip-domain serine protease* gene was thought to be involved in the activation of the Toll-pathway (Kanost and Jiang, 2015), it was expected that it would follow a similar pattern of expression as the other genes in this study that are involved in, or produced by, the Toll-pathway. However, *clip-domain serine protease* expression does not follow the same pattern. This contradictory result could indicate that the *clip-domain serine protease* was not performing the role originally thought. There are many serine protease genes in the FAW genome, with 113 and 86 digestive serine proteases identified in the rice and corn strain respectively, and 16 clip-domain proteases with potential immune functions (Gouin et al., 2017). In a non-model organism, it is hard to determine the specific function of a gene without functional validation using RNAi or CRISPR, and this could be an important next step for the *clip-domain serine protease* gene. In the model organism *B. mori* there is a very closely related serine protease gene (97% nucleotide match) that is involved in the activation of the cecropin B promotor (Taniai and Imanishi, 2004). Cecropin B is an antimicrobial peptide produced by the Toll-pathway in response to gram-negative and gram-positive bacteria (Tsakas and Marmaras, 2010). Therefore, it is very likely that this *clip-domain serine protease* in FAW has a role in immunity and this role may be through activating antimicrobial peptides instead of activating the Toll-pathway.

5.5.2 Flight changes the Toll-pathway response in SfMNPV-challenged fall armyworm differently in males and females.

There was a significant three-way interaction between flight behaviour, viral challenge, and sex on gene expression. The role infection plays in these three-way interactions is particularly interesting as it suggests viral challenge may change flight behaviour in each sex differently. Infection has been shown to change flight behaviour in

parasitized (*Nosema ceranae*) honey bees (*Apis mellifera*), with infected bees undertaking fewer, shorter flights compared to non-infected bees (Alaux et al., 2014). The positioning of the challenged groups on the PC axes (Fig. 5.3) further supports the theory that SfMNPV challenge may lead to behavioural flight changes as both challenged males and females are positioned in different directions on the PCA plot compared to non-challenged males and females. Understanding this relationship may be better explained by separating out the key variables contributing to each PC. This reveals that challenged FAW flew shorter flights covering less distance compared to non-challenged FAW, however, the number of flights and speed was very similar across the challenged and non-challenged groups (Fig. 5.5). Additionally, there were differences between the sexes in both treatment groups, with on average females flying more than males, with the number of flights, distance, and duration all higher, however, the mean speed was very similar in both males and females. Similar findings have been found in the migratory Monarch butterfly (*Danaus plexippus*), as males flew shorter distances than females, and parasitized butterflies flew shorter distances compared to healthy butterflies (Bradley and Altizer, 2005).

The analysis of flight behaviour revealed that the two sexes responded differently to viral challenge at the molecular level. Supporting our hypothesis, in the flown challenged and non-challenged groups, all three genes that were significantly affected by sex (*defensin*, *MYD88* and *clip-domain serine protease*) were expressed at higher levels in females compared to males. This suggests that females prioritised a higher baseline level of immunity than males. The reverse pattern was observed for *cactus*, however, this gene is a negative regulator of the Toll-pathway so it's lower expression could potentially suggest higher immunity (Belvin and Anderson, 1995; Ferrandon et al., 2007). Overall, these findings suggest that irrespective of viral challenge, flown females have higher expression of Toll-pathway genes compared to males. Sex differences in Toll-pathway immunity have previously been observed in fruit flies (*Drosophila melanogaster*) (Duneau et al., 2017; Belmonte et al., 2020), with strong dimorphism observed between the sexes both constitutively and in response to an immune challenge, with males having higher survival rates when immune-challenged, and higher constitutive expression of Toll-pathway genes than females (Duneau et al., 2017). Similarly, in the tobacco budworm (*Heliothis virescens*) (not-flown) uninfected males invested more in immune maintenance compared to females (i.e., higher baseline expression of immune related genes) (Barthel et al., 2015). These studies contradict the findings of the current study because for three of the genes (*defensin*, *MYD88* and *clip-domain serine protease*) expression was higher in both non-challenged and

challenged females compared to males. However, previous findings in other polygynous insects also found males had lower immune function than females (Nunn et al., 2009; Wilson and Cotter, 2013).

On the other hand, within-sex comparisons show that Toll-pathway gene expression responds differently in each sex following flight. Challenged females had lower expression of all four Toll-pathway genes compared to non-challenged females, whereas challenged males had higher expression for *cactus* and *MYD88* and very little change for *defensin* and *clip-domain serine protease* compared to non-challenged males. This response of challenged males and females was the reverse of the hypothesis that challenged males would decrease immune gene expression compared to non-challenged whilst the respective female groups would increase it. Therefore, challenged males invested more into immunity when flown compared to challenged females, which suggests higher levels of immune investment during the stress-inducing behaviour of flight.

These differences in immune investment between virus-challenged flown females and males are likely due to females needing to contribute more energy to reproduction, and consequently having less resources to invest elsewhere (Schwenke et al., 2016). Thus, females had to compromise between flight, immunity, and reproduction, and did so by lowering immune gene expression. Consequently, by manipulating investment into immunity in this way they were able to maintain a stronger flight ability (i.e., flying further) than challenged males were. These trade-offs are important as both immunity and flight are energy-demanding activities (Kammer and Heinrich, 1978; Suarez, 2000; Ardia et al., 2012). Reductions in immunity due to flight have been found to occur in other insects, such as reduced lysozyme activity in crickets (*Gryllus texensis*) (Adamo and Parsons, 2006). One explanation for these trade-offs is that energy for both flight and immunity can be sourced from carbohydrates and lipids in insects (Teo et al., 1987; Dolezal et al., 2019; Somerville et al., 2019).

The observed sexual dimorphism in immunity (i.e., flown males exposed to SfMNPV increase immunity whereas females decrease it) could also be explained by the hypothesis of the susceptible male. This hypothesis suggests that there is female mate-choice for males with higher immunity, and that this higher immunity may be identifiable by females due to physical characteristics (Hamilton and Zuk, 1982). In insects, this could be visible in subtle ways such as increased melanin leading to darker cuticles in *Drosophila*, or by affecting secondary sexual traits, such as leg bristles and combs (Belmonte et al., 2020). This has not

yet been studied in FAW, however, the results presented here provide evidence of strong sexual dimorphism between the sexes in immunity; higher immune investment in males in stressful conditions (i.e., flight and viral challenge) whereas under the same conditions females reduced immune investment. This increased immunity in males came at the cost of reduced flight ability compared to females. Increasing immune investment could benefit males by increasing their likelihood of mating success following flight, because higher immunity and lower viral loads could increase attraction to females. Further work is needed to investigate this relationship in more detail to confirm if immunity and viral loads have an impact on mating success in FAW.

Clip-domain serine protease was expressed differently to the other three genes when comparing challenged and non-challenged flown FAW. There was lower expression of *clip-domain serine protease* in challenged males compared to non-challenged males, whereas for *cactus, defensin* and *MYD88* expression was higher in challenged males. In females, there was a decrease in expression of *cactus, defensin* and *MYD88* when challenged, whereas expression levels of the *clip-domain serine protease* remained at a very similar level irrespective of viral challenge. This *clip-domain serine protease* gene was selected as it showed a significant interaction between flight and sex in challenged FAW, with it being up-regulated in females and down-regulated in males in response to flight (chapter 4). The reason for the response being different in this study is unknown, however, it could be due to population variation as the previous study (Chapter 4) used corn strain FAW collected in Brazil (South America), whereas the current study (Chapter 5) used the invasive FAW collected in Malawi (Africa). There is increasing evidence of differences between these two populations, with the population in Africa being a hybrid of the corn and rice strains, whereas hybrids are rare in South America (Zhang et al., 2020). Alternatively, other studies observed variations in immune responses with slight variations in protocol, such as diet composition (Cotter et al., 2019) or method of disease entry (Avadhanula et al., 2009; Ferreira et al., 2014). However, the significant three-way interaction between flight behaviour, viral challenge and sex suggests that during flight, *clip-domain serine protease* plays an important role, and future work should investigate this relationship in more detail using functional genomics.

These sex-specific differences were apparent only when comparing flown moths, but not when comparing flown and not-flown moths. Some significance may have been lost when comparing flown to not-flown due to the differences in model composition and the relatively small sample size. Alternatively, this could mean that the sexual dimorphism

becomes more apparent following the stress of flight and viral challenge, which led to the different patterns in expression, resulting in the observed three-way interaction. Different migratory strategies between males and females may have played a role in the sex-specific differences observed in this study, for example, in *S. exempta* males have shorter pre-reproductive periods compared to females and are likely to continue migratory flights after becoming sexually mature (Wilson and Gatehouse, 1993). Consequently, energy demands for investment in reproduction would differ between the sexes based on whether they had reached sexual maturity or not. However, in another closely-related migratory moth, *Spodoptera exigua*, sex had no impact on flight distance, velocity or duration on moths flown on flight mills between 2 and 5 days old (Jiang et al., 2010). Previous work on flight activity and its impact on immunity (Fritzsche McKay et al., 2016) and gene expression (Jones et al., 2015) in migratory Lepidoptera has focused only on females. This study highlights the need to further investigate sex-specific differences in migratory Lepidoptera.

Overall, female FAW may invest more in immunity than males, however, when females are faced with both an immune challenge and flight they favour other costly traits such as flight ability or reproduction. On the other hand, males invested less in immunity, yet, when needing to distribute energy between immunity and flight they invested more in immunity at the cost of a reduced flight ability. These responses could be explained by Bateman's principle, which is that females often invest more into evolutionary strategies related to longevity, whereas males often favour shorter lives with increased opportunities for reproduction (Bateman, 1948; Rolff, 2002). To put this into context of this study, challenged FAW females have a higher baseline immunity for longevity but decrease immune gene expression during long distance flights to migrate further and reproduce, whereas challenged males have a lower baseline immunity, but when necessary, they will increase immunity and undergo a shorter flight to find a mate and reproduce.

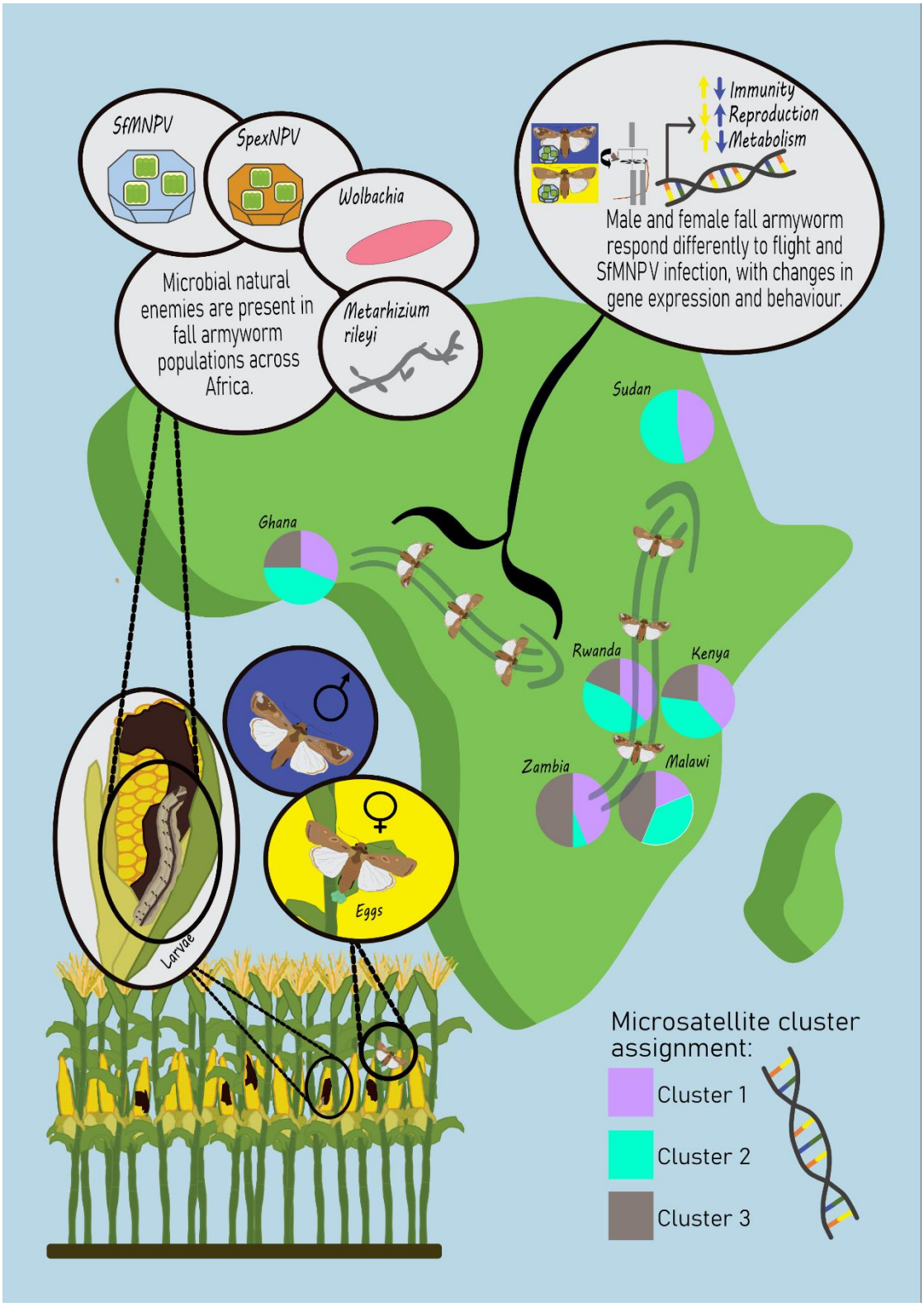
5.5.3 Conclusion

This study found strong evidence of flight directly affecting Toll-pathway gene expression following SfMNPV challenge in FAW. Overall, these findings show that challenged FAW will be able to prioritise both immunity and flight to maintain an immune response. However, males and females respond differently to flight and SfMNPV challenge, with evidence that these differences may be linked to Bateman's principle leading to differential investment in immunity and reproduction.

When considering immune gene expression, it is important to note it may not directly relate to functional immunity, however it can give a good indication of the molecular processes. The effects of gene expression on immunity are hard to determine, for example, an increase of immune-related genes in the Glanville fritillary butterfly did not lead to increased survival when faced with an immune challenge (Woestmann et al., 2016). Similarly, other work has shown that immune gene expression does not always directly relate to functional immunity in *Spodoptera littoralis* (Cotter et al., 2019). Unfortunately, due to the nature of how the FAW are attached to the flight mills, it is not possible to continue the experiment to assess survival rates. However, many studies in insects have used gene expression to gain an insight into immunity and determine how insects respond to infection (Zou et al., 2006; Xi et al., 2008; Barthel et al., 2015; Sun et al., 2017). Therefore, it is discussed here how gene expression was affected by viral challenge and flight to further contribute to this knowledge.

This is the first study of its kind to look at how gene expression is affected by the challenge of viral infection in each sex of a global insect pest both between not-flown and flown moths and within flown moths. The findings show that gene expression is highly variable within flown moths, and that flight behaviours such as speed, distance, number of flights and duration all contribute to this. This interaction is important to understand considering that flight behaviours greatly contribute to the spread of this invasive species. Understanding more about how viral challenge can influence flight and immunity contributes to current knowledge of how SfMNPV, both as a natural enemy and as a potential biocontrol agent, may influence the movements of this crop pest in those that are able to survive infection.

Chapter 6 – Discussion and Summary



6.1 Thesis overview

Overall, throughout this PhD the aim was to decipher the complex interactions between migration and disease, using an invasive crop pest we urgently need to learn more about, the fall armyworm (*Spodoptera frugiperda*), as the study organism. This work has greatly enhanced our understanding of the fall armyworm in Africa, and the results are relevant to many other migratory insects, particularly other migratory Lepidoptera.

The first research chapter (Chapter Two) fills a large gap in the current knowledge of fall armyworm by identifying which microbial natural enemies are present in Africa. This confirms that four are present in fall armyworm at varying prevalences across Africa: SfMNPV, SpexNPV, *M. rileyi* and *Wolbachia*. Furthering this, an in-depth look at SfMNPV reveals that this variation could be due to temperature, rainfall, elevation, growing season, and the time since fall armyworm first invaded each country.

To understand fall armyworm population structure in its introduced region Chapter Three uses microsatellite analysis on larval samples collected in six African countries. This novel approach reveals that high levels of genetic mixing are occurring between populations of fall armyworm in Africa, however, some evidence is presented that suggests SfMNPV distribution might be influenced by which populations mix more frequently.

To help interpret the spatial variation in natural enemy prevalence and population level clustering potentially linked to SfMNPV, gene expression analysis is combined with flight mill experiments to identify potential trade-offs between flight and immune response. Chapter Four uses RNA-sequencing to take an in-depth look into gene expression in each sex following SfMNPV-challenge and flight to determine how each sex responds to these stresses. This revealed both similarities and differences in gene expression related to reproduction, immunity, metabolism, and ROS protection, highlighting that compromises between these areas were occurring which could influence migratory capacity. To investigate this in more detail, in Chapter Five RT-qPCR is used to determine the response of the immunity-related Toll-pathway. This provided some evidence that fall armyworm can control immune gene expression to favour flight, and confirmed that males and females invest differently in immunity when exposed to the stressors of viral challenge and flight.

Here, all of these results are discussed to consolidate the knowledge, focusing on how these findings contribute to current understanding of the fall armyworm, migratory ecology, and molecular biology.

6.2 Increased understanding of the fall armyworm in Africa

6.2.1 Covert microbial natural enemies are present in fall armyworm in Africa.

This thesis has increased understanding of the prevalence of natural enemies in fall armyworm in Africa. This is important as many infections are asymptomatic, so understanding prevalence based on quantifiable factors (e.g., weather) can help understand the risk posed to crops by fall armyworm to guide the amount of pesticide that might be needed. Furthermore, this work has highlighted that natural enemies in invasive species may be at higher levels than originally thought, and that they could be rapidly exposed to novel pathogens that are native to the invaded region. This contributes to current understanding of the enemy release hypothesis in invasive insects and provides further evidence that this hypothesis may not be widely applicable to all invasive crop pests in relation to microbial natural enemies.

Natural enemies have an important role in controlling crop pests, they can naturally reduce population size of pests leading to improved crop yields to protect against food shortages and reduce economic losses (Cugala et al., 2006). When an invasive species first moves into a new area the enemy release hypothesis suggests that the invasive species will be exposed to fewer natural enemies in the new environment as it has left its native enemies behind, and potential enemies in the new environment have not yet encountered the new host (Cornell and Hawkins, 1993; Torchin and Mitchell, 2004). This can have important implications for the spread of invasive insects globally. The loss of microbial natural enemies, including the bacteria *Wolbachia,* RNA viruses and fungal pathogens, in the invasive fire ant (*Solenopsis invicta*) are likely to have contributed to its rapid spread in three continents (Asia, Australasia and North America) (Yang et al., 2010). Therefore, it is important to learn what natural enemies are present in an invasive species' new environment, and Chapter Two helped to answer this question for the fall armyworm (*Spodoptera frugiperda*) in Africa as well as expand our knowledge of how rapidly populations of microbial natural enemies can respond to non-native species.

This research revealed that four microbial natural enemies are present in Africa, these are *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV), *Spodoptera exempta nucleopolyhedrovirus* (SpexNPV), *Metarhizium rileyi* and *Wolbachia*. The prevalence in Africa varied for each pathogen: SfMNPV 0 to 30%, SpexNPV 0 to 13%, *M. rileyi* 0 to 11% and *Wolbachia* 0 to 4%. Despite fall armyworm only being recorded in Africa from 2016, these prevalence levels are similar to microbial natural enemies that are found in the native

region of the fall armyworm, such as *M. rileyi* (0 to 17%) and SfMNPV (11%) in Mexico (Lezama-Gutiérrez et al., 2001; Virgen et al., 2013).

M. rileyi is present in invasive fall armyworm populations in China and India, and this study was able to confirm that the fungal infections naturally occurring in fall armyworm in Zambia were *M. rileyi* (Mallapur et al., 2018; Gichuhi et al., 2020; Food and Agriculture Organisation, 2018; Zhou et al., 2020; Firake and Behere, 2020). The confirmed presence of *M. rileyi* as a natural enemy of fall armyworm in Africa is significant as it can have an important role in controlling field populations. *M. rileyi* and *SfMNPV* were responsible for around 50% of fall armyworm mortalities in the field in North East India, and coinfections of both these pathogens can increase mortality as they are able to infect the host simultaneously (Mallapur et al., 2018; Souza et al., 2019).

In the native region of fall armyworm (North and South America), SfMNPV is known to occur naturally where it can be present at high levels within populations (Gómez et al., 2013; Cipriano et al., 2013; García-Banderas et al., 2020). This thesis provides the first report that SfMNPV is present in east Africa. Considering that fall armyworm were not known to be in Africa prior to 2016, it is very likely that some of the first fall armyworm to arrive carried SfMNPV with them, and then continued to spread SfMNPV into China (Lei et al., 2020). This confirmation that SfMNPV is already present in Africa will hopefully support applications to register SfMNPV as a biopesticide, a process that is current being carried out in seven sub-Saharan countries (Bateman et al., 2018; Bateman et al., 2021). This would have the advantage of reducing insecticide use, which is safer for farmers, and will have little effect on non-target species such as the parasitoids which act as natural enemies (Armenta et al., 2003; Gómez et al., 2013).

Nucleopolyhedroviruses (NPVs) are generally highly species-specific, which is one of the reasons they make excellent candidates for biocontrol agents. However, some NPVs are known to have wide host ranges, such as *Autographa californica multicapsid* NPV and *Mamestra brassicae multicapsid* NPV (Goulson, 2003). Prior to this work, SpexNPV has only been known to infect one host, the African armyworm (*Spodoptera exempta*), however genetic screenings identified SpexNPV in fall armyworm collected in Africa (Chapter Two). It is likely that host spillover occurred and SpexNPV was picked up naturally in the field by fall armyworm when they encountered African armyworm (e.g., via sharing host plants). However, more work needs to be carried out to understand the effects that SpexNPV has on

fall armyworm. It needs to be established whether SpexNPV biocontrol agents could potentially target both species, and the virulence of SpexNPV in a non-target host.

There is little known about the importance of host spillover events in the presence of natural enemies in invasive crop pest populations, because much of the research has focused on the effects of pathogens spilling over from invasive species to native, beneficial insects (Vilcinskas, 2019; Manley et al., 2015). Spillover events are known to cause problems to native insects, such as spillover of the fungal pathogen *Nosema thompsoni* to native ladybirds (*Coccinella septempunctata*) from the invasive harlequin ladybird (*Harmonia axyridus*) (Vilcinskas et al., 2013), and spillover of *Nosema ceranae* from invasive honeybees (*Apis mellifera*) to native stingless bees (*Tetragonula hockingsi*) (Purkiss and Lach, 2019). Spillover events in insects can be considerable, and could contribute to increased natural enemies in invasive pests. Evidence from the invasive common wasp (*Vespula vulgaris*) suggests its range of microbial natural enemies is similar in its invaded range to its native range because of the spillover of generalist pathogens from native social insects (e.g., other Hymenoptera or *A. mellifera*). Together with our findings in fall armyworm, this suggests that pathogen spillover between invasive and native insects could be an important factor for the abundance of natural enemies in invasive species. Further work should consider potential pathogen spillover events in all regions that fall armyworm has recently reached, as well as encourage studies on the natural enemies of other invasive crop pests to screen for those that may spillover from native hosts.

The enemy release hypothesis proposes that escape from natural enemies might be one of the key factors behind the rapid spread of invasive species. This has been shown to occur in many different taxa, with invasive crustaceans, mammals, amphibians, and birds all having lower parasitism rates than native species within the same ranges (Torchin et al., 2003). Furthermore, the rapid spread of the invasive harlequin ladybird (*Harmonia axyridis*) has been partially attributed to its escape from natural enemies in its new range, with it having lower rates of parasitism in the UK compared to native the native ladybird *Coccinella septempunctata* (Roy et al., 2016). Therefore, it was hypothesised that the rapid spread of the fall armyworm may be partially due to fewer natural enemies in Africa, however, the findings presented in Chapter Two suggest that fall armyworm might not have escaped natural enemies for long. Firstly, in its native range in Mexico, SfMNPV was the most common natural enemy (Virgen et al., 2013) and the presence of SfMNPV in Africa suggests that fall armyworm did not escape SfMNPV as it was transported with the founding population. Secondly, the time since fall armyworm had first been recorded in Africa was not

a significant factor in describing the prevalence of SfMNPV, suggesting that there had been little change in prevalence over the time since the samples were collected (ranging over a two-year period from January 2017 to June 2019). Furthermore, SpexNPV and *Wolbachia* have very rapidly established in multiple populations of fall armyworm in Africa. This means fall armyworm was quickly exposed to novel natural enemies in its new range alongside a natural enemy from its native region. These findings are supported by previous work showing that the prevalence of natural enemies is low in newly invaded territories but increases quickly as population density increases (Hajek and Tobin, 2011). Microbial natural enemies (*Lymantria dispar* NPV and *Entomophaga maimaiga*) were present in recently established invasive populations of the gypsy moth (*Lymantria dispar*), and very rapidly moved into new areas as the gypsy moth spread (Hajek and Tobin, 2011). A similar pattern could explain why the time since fall armyworm had been recorded in each country was shown to be linked to SfMNPV prevalence both in the PCA analysis and within Malawi, even though the time since fall armyworm had first been recorded in Africa was not significant. The enemy release hypothesis has also been rejected in other invasive insect species, with similar parasitoid rates occurring in both native (*Cycloneda sanguinea, Eriopis connexa* and *Hippodamia convergens*) and invasive (*Harmonia axyridis*) ladybirds in Brazil (Paula et al., 2021).

Another explanation for the lack of enemy release hypothesis applying to microbial natural enemies in the fall armyworm is that there are closely related species present in Africa (e.g., African armyworm, beet armyworm (*Spodoptera exigua*)). The more closely related an invasive species is to native species the more likely they are to be targeted by natural enemies present in the invaded area (Harvey et al., 2012). It was found that the invasive light brown apple moth (*Epiphyas postvittana*) had a similar abundance of natural enemies in areas it had invaded (California) compared to its native region (Australia), and many of these natural enemies were associated with closely related tortricid species that shared host plants (Bürgi and Mills, 2014). Furthermore, our proposition that two natural enemies (SpexNPV and *Wolbachia*) were present in fall armyworm due to spillover from African armyworm further supports these previous findings, as these spillover events were probably aided by host relatedness. The findings presented here support the growing evidence that the relatedness of invasive species to native ones may influence the extent to which invasive species are able to escape natural enemies due to an increased likelihood of pathogen spillover events. This highlights the importance of identifying microbial natural

enemies in both native and invasive crop pests as understanding this could improve pest management for invasive species.

The occurrence of these spillover events of pathogens from the African armyworm into fall armyworm implies that the species must frequently encounter each other, leading to novel interactions and competition for native pest species. As well as the African armyworm, studies have shown that interspecies competition with the fall armyworm and other stemborers is highly likely, including *Chilo partellus, Busseola fusca* and *Sesamia calamistis* (Sokame et al., 2020a; Sokame et al., 2020b). Due to the likelihood of these species coming into close contact via sharing ranges and host plants, it is possible that further spillover of natural enemies may have occurred, or it could occur in the future. Future work should aim to identify whether any other spillover events have taken place, both from the native species to the invasive pest and vice versa.

Apparent competition can occur when species share natural enemies, and the presence of both species has the potential to increase the natural enemies population (Holt and Lawton, 1994). This apparent competition arises from interactions between the hosts competing for limited resources and trading-off between defence mechanisms and competitive ability (Holt and Lawton, 1994). This can affect the populairon dynamics of insect pest species, with the predator big-eye bug (*Geocoris puncitipes*) preferentially feeding on the pea aphid (*Acyrthosiphum pisum*) over the corn earworm (*Helicoverpa zea*), thus reducing aphid populations whilst increasing corn earworm populations (Eubanks and Denno, 2000). However, other work has shown that interspecific resource competition has a greater impact when competing aphid hosts (*Acyrthosiphum pisum* and *Megoura viciae*) share a parasitoid natural enemy (*Vicia faba*) (Jones et al., 2009). Considering this thesis revealed that spillover events had occurred it is likely that the fall armyworm shares a range of natural enemies with native pest species, which adds a further interaction to consider when determining the effects that natural enemies have on insect pest assemblages in Africa.

To date, little is known about what effects shared microbial natural enemies could have on insect pest species and the competition between them. There is evidence that shared hosts could increase the prevalence of fungal entomopathogens through increased transmission and dispersal, especially when the pest species share parasitoid or predatory natural enemies (Roy and Pell, 2000). The results presented in this thesis support this as it is highly likely that the fall armyworm was infected with *M. rileyi* due to close contact with the

African armyworm, or another stemborer, when sharing host plants. Computational modelling has shown that the impact of specialist microparasites, such as the viruses studied in this thesis, on species competition can vary considerably (Fenton and Brockhurst, 2008). These effects include enabling co-existence by reducing the stronger competitor so the weaker competitor can persist and facilitating the spread of invasive species by reducing native competitor species (Fenton and Brockhurst, 2008). Disease mediated apparent competition affecting insect populations has been shown to occur in flour beetles in laboratory experiments, with the weaker competitor *Tribolium confusum* only able to outcompete *Tribolium castaneum* in the presence of the sporozoan parasite *Adelina tribolii* (Park, 1948; Hudson and Greenman, 1998). Now this thesis has identified shared microbial natural enemies between pest species in Africa, future work could investigate the potential impacts of these interactions and if they are contributing to species assemblages and populations dynamics.

The confirmed presence of microbial natural enemies means that future work can focus on understanding their effects on fall armyworm populations, such as virulence and mortality rates in the field. These natural enemies could then be incorporated into IPM programmes, and potentially make a significant contribution to achieving the United Nations Sustainable Development Goals such as reducing food shortages, improving food security, and promoting sustainable agriculture. An indirect benefit of natural enemies is the reduced need for biochemical inputs such as insecticide sprays which benefits the farmer economically through improved yields and reduced pesticides costs, as well as lowering carbon dioxide (CO_2) emissions, and preventing losses of non-target species (Zhang and Swinton, 2009; Heimpel et al., 2013; Jepson et al., 2014; Andersson and Isgren, 2021). Thus, by increasing our understanding of the prevalence of natural enemies in Africa it means that farmers may be able to use this information to improve fall armyworm pest control, leading to less damage and consequently higher yields.

Another reason for understanding the prevalence of natural enemies is that their presence can change the migratory capacity of Lepidoptera. The impact of natural enemies on migration in Lepidoptera is well studied in the monarch butterfly (*Danaus plexippus*) and its parasitic natural enemy *Ophryocystis elektroscirrha*. Infected monarchs are unable to migrate as successfully as uninfected monarchs due to a loss of flight ability and migratory culling (i.e., infected individuals removed from the population due to being unable to survive) (Bartel et al., 2011; Bradley and Altizer, 2005). Laboratory experiments using flight mills have shown that similar effects of infection on flight are observed in fall armyworm,

with males having reduced flight ability following exposure to SfMNPV (Pearson, 2016 and Chapter Five). Therefore, it is likely that wild populations of fall armyworm will be experiencing similar effects of covert infection on their migratory capacity and this could have important consequences for the movements of fall armyworm. The high prevalence of natural enemies such as SfMNPV could lead to reductions in the number of fall armyworm undertaking long distance flights across the continent alongside an increase in the number of resident populations which would lower the risk of insecticide resistance spreading. Whilst this hypothesis needs further work (e.g., by monitoring movements and disease prevalence in field populations of fall armyworm), Chapter Three did investigate population structure and disease to see if there was any structuring between populations with and without SfMNPV present (discussed further in section 6.2.2 and 6.3).

In conclusion, this research has greatly contributed to our knowledge of microbial natural enemies in fall armyworm in Africa. It has both identified novel natural enemies and considered how key environmental features could help to explain disease prevalence. Similar research in other countries that fall armyworm have been introduced too now needs to be carried out to improve understanding of fall armyworm natural enemies globally. Furthermore, similar studies to identify microbial natural enemies in other invasive pest species will be necessary to further improve understanding of how the enemy release hypothesis contributes to their spread.

6.2.2 Fall armyworm populations are regularly mixing across in Africa.

Understanding population structure in fall armyworm is essential to revealing how fall armyworm might be moving around the newly invaded areas, especially given its ability to undertake long distance flights (Johnson, 1987; Westbrook et al., 2016). Population structure and movements have important implications for key features such as insecticide resistance and potentially, for the patterns of disease prevalence that were identified in Chapter Two. These results greatly enhanced current understanding of population structure in fall armyworm by overcoming challenges around unidentifiable strains and low genetic variation in the established genetic markers by utilising microsatellite markers.

Since fall armyworm first invaded Africa, many studies have aimed to determine the population structure of fall armyworm by using the *mitochondrial cytochrome oxidase I* (COIB) and the Z-chromosome-linked *Triosephosphate isomerase* (Tpi) markers that are both well-established for use in fall armyworm in its native region (Nagoshi et al., 2015; Westbrook et al., 2016; Nagoshi and Meagher, 2016). In North and South America, these

markers can differentiate between populations and identify genetic structure based on strain (rice or corn) distribution and haplotype diversity (Nagoshi et al., 2015; Westbrook et al., 2016; Nagoshi and Meagher, 2016). However, in invasive fall armyworm populations this led to a lot of confusion around which strain was present, with the COI marker reporting that most individuals were the rice strain, whereas the Tpi marker was reporting most individuals as the corn strain, a pattern that was also identified in Chapter Two (Nagoshi et al., 2017b; Nagoshi et al., 2018b; Nagoshi et al., 2019b; Nagoshi et al., 2020; Nayyar et al., 2021). Further research using genetic sequencing eventually solved this discrepancy by confirming that the invasive fall armyworm populations were made up of a hybrid of the corn and rice strains (Zhang et al., 2020).

Previously, the high levels of genetic diversity in the COIB and Tpi markers meant that population movements of fall armyworm in their native regions could be determined. This approach was used to determine diversity in the Americas, including in Brazil, Puerto Rico, Florida, Texas, and Alabama (Nagoshi et al., 2010; Westbrook et al., 2016). Using this approach it was possible to determine where long-distance migratory flights were occurring and that some fall armyworm populations were becoming more isolated, such as those in Puerto Rico that originated from populations in Brazil (Nagoshi et al., 2010). However, in Africa there was very little genetic diversity in fall armyworm in either the COIB or Tpi markers, with both this thesis and previous work only able to identify two COIB haplotypes (COIB-R and COIB-H4) whereas there are five known COIB haplotypes in native fall armyworm (Nagoshi et al., 2019a; Nagoshi et al., 2019b). Similarly, low genetic diversity in a COI marker was found in bed bugs (*Cimex hemipterus*) whereas microsatellites had greater genetic diversity and were more informative when determining the population structure of this invasive pest in Malaysia and Singapore (Wan Mohammad et al., 2020).

The lack of genetic diversity observed in the invasive fall armyworm meant it was hard to determine whether populations were mixing. There were reports that there was potentially an east-west divide between fall armyworm populations in Africa (Nagoshi et al., 2018b), or no clear pattern of division between countries across the continent (Nagoshi et al., 2019b). Considering the importance of determining population structure and mixing, in Chapter Three we used microsatellites to see if there would be greater levels of genetic diversity that could help determine population structure of fall armyworm across Africa. Microsatellites had previously been designed for fall armyworm, and had been used to determine genetic diversity and structure of fall armyworm populations in Paraguay and Brazil (Arias et al., 2019). Comparable levels of genetic diversity for microsatellites were

found in this study, with similar allele ranges observed in native (3 to 15) and invasive (3 to 13) fall armyworm (Arias et al., 2019; Pavinato, 2013). This meant that population structure could be investigated in more detail than with COIB and Tpi markers.

Genetic diversity in microsatellites revealed that populations of fall armyworm across Africa appear to be mixing frequently with others. Based on the patterns of differentiation observed, it is hypothesised here that fall armyworm in Africa are undertaking long-distance flights aided by the movement of dominant winds. These wind patterns largely follow the inter-tropical convergence zone (ITCZ), meaning the winds are generally south-easterly north of the equator and north-easterly south of the equator (Rose et al., 2000). Supporting this theory is a large amount of literature which has investigated the movements of a similar species in Africa, the African armyworm, which also follows these dominant winds in its migratory routes across the continent (Tucker et al., 1982; Rose et al., 1985; Tucker, 1994; Rose et al., 2000).

On the other hand, microsatellites identified populations of fall armyworm in Africa that were more genetically distinct than other others (e.g., Sudan), whilst there was less evidence of this genetic structuring based on the mitochondrial markers (Chapter Three). This supported the hypothesis that microsatellites would be more informative for studying population structure in invasive fall armyworm. Similarly, in the spotted lantern fly (*Lycorma delicatula*) microsatellites detected further subdivision of populations in China and more individual migrants compared to mitochondrial markers (Zhang et al., 2019). Therefore, microsatellites can be better than mitochondrial markers when being used to detect fine-scale features in populations such as dispersal patterns and clustering (Zhang et al., 2019; Wan Mohammad et al., 2020).

Based on the identification of genetically distinct populations in fall armyworm it is proposed that whilst some fall armyworm are undergoing long-distance flights others may be forming resident populations, so less mixing with other populations occurs. Facultative migration (i.e., when individual migrants can choose whether to migrate) in fall armyworm has been observed in parts of their native region, with some fall armyworm populations from regions in South America and the Caribbean forming resident, genetically-segregated populations (Nagoshi et al., 2017a). What is driving this segregation in South American populations is unclear, but facultative migration has similarly been observed in the invasive cotton bollworm (*Helicoverpa armigera*), meaning that some populations are resident whereas other undergo long-distance migratory flights (Jones et al., 2019; Jyothi et al.,

2021). This partial migration is likely to be driven by a range of factors such as habitat quality, geographic features, temperature, and population density (Dingle, 2014; Jones et al., 2019; Jyothi et al., 2021). One of the reasons for the establishment of resident populations of invasive fall armyworm is likely to be that the tropical African climate would enable year-round survival and reproduction as long as host plants could be found (Early et al., 2018; Cokola et al., 2020). Considering that fall armyworm is a generalist species, with 353 known host plants in the Americas, then it is likely that small, resident populations would be able to find suitable hosts (Montezano et al., 2018). Furthermore, migratory behaviour can rapidly be lost by Lepidoptera in the laboratory once selection for migration is removed, with migratory traits declining within a year in laboratory populations of the Monarch butterfly (*Danaus plexippus*) (Davis et al., 2020). Therefore, it is likely that if the selective pressure to migrate was lost in the wild then the costs of migration would soon outweigh the benefits of undertaking long-distance migratory flights (Roff and Fairbairn, 2007a; Davis et al., 2020). This switch to more resident populations could occur rapidly due to the strong genetic component involved in the inheritance of migratory flight, which has been observed in the African armyworm and the marmalade hoverfly (*Episyrphus balteatus*) (Wilson and Gatehouse, 1992; 1993; Dällenbach et al., 2018).

Previous work on mitochondrial markers suggested that there was only one introduction event of fall armyworm into Africa (Nayyar et al., 2021; Zhang et al., 2020; Nagoshi et al., 2019a). The findings presented in Chapter Three using both mitochondrial markers and microsatellites support this theory. This contrasts with what has been observed in other invasive Lepidoptera where multiple introduction events have occurred, with genetic differentiation identified by microsatellite analysis in widespread populations of the non-migratory codling moth (*Cydia pomonella*) in China suggesting multiple colonisation events (Men et al., 2013). Similarly, mitochondrial markers in cotton bollworm in Brazil revealed that at least two introduction events resulted in successful establishment (Tay et al., 2013). One possible explanation for a single introduction event occurring for fall armyworm in Africa is that fall armyworm has only been in Africa for a very short amount of time (around 5 years), thus reducing the likelihood of successive introductions occurring since that first introduction. Multiple introductions have occurred for other invasive Lepidoptera, however the pests have been present for much longer in their invaded regions increasing the likelihood of successive introductions, such as the codling moth that first invaded China in 1950 (Men et al., 2013), and cotton bollworm was first identified in South America in 2008 (Jones et al., 2019).

The rapid spread of insecticide resistance in the cotton bollworm, a migratory invasive pest, is well documented, and is one of the factors that makes it difficult to control. Understanding the migratory movements of cotton bollworm can help farmers to control this pest through knowing what insecticides it is likely to be resistant too (Armes et al., 1996; Xiao et al., 2017; Buès et al., 2005; Jones et al., 2019). Therefore, it is important to consider our increased understanding of population structure in relation to known insecticide resistance patterns and alleles in invasive fall armyworm. It is known that fall armyworm in Kenya, Malawi, Benin, and Uganda have a high frequency of alleles that can give resistance to synthetic insecticides including organophosphates and carbamates (Boaventura et al., 2020; Yainna et al., 2021). Furthermore, in China, fall armyworm carry alleles for resistance to organophosphate, pyrethroid and diamide insecticides, thus it is likely that these resistances are present in Africa (Zhang et al., 2020). The potential for emerging insecticide resistances is high in fall armyworm due to rapidly evolving resistance mechanisms soon after insecticide use. One example is the evolution of Bt resistance emerging independently in two geographically distant populations of fall armyworm in the Americas (Puerto Rico and Brazil) (Nagoshi et al., 2017a). Considering we now know that the fall armyworm populations in Africa are largely panmictic, the risk of insecticide resistance rapidly spreading across the continent, and potentially further into Asia, is very high. Therefore, this work has highlighted the need for regular monitoring of fall armyworm populations and shown that the application of microsatellites to understand population structure and movement can aid such monitoring.

As well as determining possible movements of fall armyworm in Africa, this thesis aimed to establish how disease could affect the flight behaviour of fall armyworm. It is known that male and female fall armyworm respond differently to SfMNPV infection and flight, with infected females maintaining flight ability whilst males fly slower and shorter distances when infected (Pearson, 2016). By using microsatellites, some evidence was found that fall armyworm infected with SfMNPV clustered together both within and between countries. There are two potential hypotheses explaining this finding. Firstly if some fall armyworm are forming resident populations then disease can increase due to the absence of migratory culling, because if a population is not undertaking long-distance flights then those infected individuals will not necessarily be removed from the gene pool (Chapman et al., 2015). The likelihood of resident populations forming in areas of Africa is high as the benefits of undergoing long distance flight might not outweigh the costs. In parts of the USA, fall armyworm are migratory to avoid freezing temperatures during winter in more northern

regions (Westbrook et al., 2016). However, temperatures in much of Africa enable year-round survival and breeding to occur (Abrahams et al., 2017; Zacarias, 2020). Therefore, the capacity and driving mechanisms behind long-distance flights could be rapidly lost without this selection pressure, similar to what has been observed in laboratory populations of migratory Lepidoptera (Davis et al., 2020). Secondly, nucleopolyhedroviruses can be passed across generations vertically meaning that the offspring of infected fall armyworm are more likely to have SfMNPV than unrelated individuals. This is similar to how SpexNPV persists in African armyworm field populations (Vilaplana et al., 2008; Vilaplana et al., 2010).

This work builds on the use of microsatellites for determining population structure, especially when genetic bottlenecks commonly associated with invasive populations means that mitochondrial DNA has low genetic diversity (Wan Mohammad et al., 2020; Zhang et al., 2019). Overall, this thesis has increased our understanding of the population ecology of fall armyworm in Africa and highlighted that whilst many populations appear to be mixing there is some evidence of resident populations forming. This can have important implications for the impact of natural enemies as we found evidence that fall armyworm with SfMNPV were more likely to be genetically clustered, meaning that the disease might increase in resident populations and help naturally control populations. Furthermore, knowing that populations are moving frequently around Africa has importance consequences for control of fall armyworm, both in terms of the risk of insecticide spreading, and to determine potential migratory routes. This could help predict cropland areas at risk of further attack on an annual basis.

6.3 How molecular biology helps to explain the interaction between disease and flight

Having established large scale population level movements of fall armyworm across Africa, and the accompanying variation in prevalence and distribution of microbial natural enemies, Chapters 4 and 5 focused on the molecular mechanisms that are driving the trade-offs and larger scale outcomes between migration and disease. Selecting the most prevalent disease, SfMNPV, this research analysed RNA to look for evidence of differences in the molecular response of males and females, and the extent to which specific immune responses differ in insects which have undertaken long distance flight.

As well as furthering our understanding of the effects of natural enemies on populations in Africa, this molecular analysis also builds on previous work that showed differences in the migratory potential of both sexes when infected with SfMNPV (Pearson,

2016). Specifically, that female fall armyworm from South America were able tolerate the virus and maintain flight ability whereas males showed weaker flight ability if infected with a virus (Pearson, 2016). In this thesis, this previous work was developed further to show that the same flight response was occurring in the African hybrid fall armyworm, and that there were molecular responses potentially driving this trade-off in flight behaviour due to differences in immunity, metabolism, and reproductive investment.

Flight is extremely energy demanding, and requires insects to change gene expression to adjust to this additional demand (Jones et al., 2015; Kvist et al., 2015). These changes in gene expression reflect the overall stresses that flight causes in both migratory (e.g., cotton bollworm) and non-migratory (e.g., Glanville fritillary (*Melitaea cinxia*)) Lepidoptera, and covers a wide range of areas, including protein production, metabolism, and muscle structure (Kvist et al., 2015; Jones et al., 2015). Much of the previous work on migratory insect flight and gene expression during flight has been carried out on females (Fritzsche McKay et al., 2016; Jones et al., 2015), however, males and females respond differently to infection and this has been shown to affect their flight behaviour, although the data varied across populations studied (Pearson, 2016). Chapters Four and Five revealed that as well as behavioural differences across the sexes, there is also evidence for key differences in the gene expression of males and females. These differences in flight ability and gene expression in moths facing an immune challenge are most likely due to differential investment in reproduction, as proposed by Bateman's principle (Bateman, 1948). Bateman's principle is that females invest more energy into reproduction than males and consequently are more selective about who they mate with, whereas males invest less in each individual mating and face greater competition from other males (intra-masculine selection) as their reproductive success depends on the frequency of these matings (Bateman, 1948). Bateman's principle occurs in Lepidoptera, for instance, in the migratory Monarch butterfly males mated whenever possible and more frequently than females, and males did not delay mating on factors such as spermatophore size or whether the female has previously mated (Oberhauser, 1988).

Supporting Bateman's principle being involved in Lepidoptera response to immunity and flight, we found that flown females have higher baseline immunity compared to males. Similarly, previous findings in other polygynous insects found males had lower immune function than females (Nunn et al., 2009; Wilson and Cotter, 2013). This could be an evolutionary mechanism in females to favour longevity, as this is more likely to increase their

reproductive success by giving them longer to find suitable mates and find higher quality habitat for their offspring.

Further evidence of the underlying mechanisms of Bateman's principle are presented in Chapter Four and Chapter Five as following flight viral-challenged females reduced immune gene expression during flight whereas males increased it. Considering that reproduction, flight and immunity are all extremely energy demanding activities it is likely that females are investing energy elsewhere during flight (e.g., prioritising flight and reproduction), whereas males are prioritising immunity over flight. This trade-off between immunity and flight could explain why females are able to fly further than males as this may increase their reproductive fitness (e.g., find better quality habitat to lay their eggs), whereas males undergo shorter flights but have higher fitness in terms of reproductive success (i.e., they have the energy to find and mate with more females so produce more offspring). This higher investment in immunity in flown, challenged males supports the susceptible male hypothesis which states that males with higher immunity are more likely to be chosen by females (Hamilton and Zuk, 1982). This means it is beneficial for infected, flown males to have high immunity as this could increase their chances of mating success following flight. Whilst this relationship has not been directly studied in Lepidoptera yet, it has been proposed that male investment in immunity may show in physical traits in insects such as melanisation or leg bristles in *Drosophila* (Belmonte et al., 2020). The differential levels of investment we identified in each sex strongly support the theory of differential energy investment between males and females, and helps to unravel the complex interactions between disease and flight. Considering the molecular evidence for the sustainable male hypothesis being active in fall armyworm, it would be interesting for further work to identify any physical features (e.g., melanisation, leg bristles) that relate to immunity and if these characteristics increase mating success in males. Overall, the differential investment in immunity between the sexes is likely to be an evolutionary strategy in migratory Lepidoptera. This strategy would allow males and females to focus their energy where and when necessary to maximise their fitness through different reproductive, immune, and migratory strategies.

Whilst this research found examples of differences in the response to disease and flight in males and females, it also found many similarities between the sexes. These similarities were in the metabolic response to the stress induced by flight, such as increased protection against reactive oxygen species (ROS) or changes in protein production. The very high energy demands of flight can trigger different sources of energy to be used in

metabolism compared to those used when an insect is not flying, some insects, such as the cotton bollworm, switch to using lipids as fuel during long distance flights (Jones et al., 2015; Wang et al., 2020). This study found support for this in fall armyworm, with strong evidence for males switching to lipid metabolism and some evidence that females did too, albeit with a much greater reliance on carbohydrates in the latter. It is thought that the metabolic rates of females and males during flight are different, with higher metabolic rates in flying females (Niitepõld et al., 2011). The differences in the fuel used for flight would support this theory that metabolism during flight is different for males and females, and these differences could also contribute to explaining why males typically fly shorter distances compared to females irrespective of viral challenge (Chapter Five and Pearson, 2016).

A lot of research has shown that the Toll immune pathway in insects responded to fungal or bacterial infection, whilst the JAK/STAT pathway responded to viral challenge (Cheng et al., 2016a; Marques and Imler, 2016). There is some evidence that the Toll-pathway has an important role in the response to viral challenge in insects too, with the upregulation of Toll-pathway components also recorded in *Drosophila melanogaster* in response to Drosophila X virus (a member of the Birnavirus family) (Zambon et al., 2005) and in *Aedes aegypti* in response to Dengue arbovirus (Xi et al., 2008). Similarly, the research presented in Chapter Four and Chapter Five strongly suggest that the Toll-pathway has a role in the immune response to SfMNPV infection in fall armyworm. This supports previous evidence that the Toll-pathway may be important in the response to viral infections in fall armyworm as an upregulation of multiple Toll-pathway genes was observed in fall armyworm larvae following infection with *Spodoptera frugiperda ascovirus 1a* (Zaghloul et al., 2020). By contrast, when *Bombyx mori* was exposed to *Bombyx mori* NPV there was no evidence of the Toll-pathway being upregulated (Cheng et al., 2016a). Therefore, generalisations in immune response between similar species might not be possible due to the complexity of insect immunity, and this highlights the importance of determining specific immune responses in a wide range of insect species.

This molecular analysis highlighted the flexibility of fall armyworm to trade-off the energetic demands of costly activities such as flight, immunity, and reproduction. The evidence from flight mills suggests that due to these trade-offs some fall armyworm face significant flight limitations due to asymptomatic viral infections in insects (Pearson 2016, Chapter Five). In migratory species, it is likely that flight limitations will affect population structure, for example, Monarch butterflies infected with a parasite showed evidence of migratory culling meaning that genetic diversity in populations with high parasite loads could

be lost following migration if infected individuals do not survive and reproduce (Altizer et al., 2000; Bradley and Altizer, 2005). However, previous work did not find any differences in SfMNPV viral load between migratory and resident populations of fall armyworm males in the USA, which does not support the theory of migratory culling lowering viral-load or prevalence in migratory populations (Pearson, 2016). However, population analysis of fall armyworm based on microsatellites showed some evidence of clustering based on SfMNPV prevalence (Chapter 3). Furthermore, in one fall armyworm population from the USA the differences in flight behaviour in response to SfMNPV infection were not apparent in flight mill experiments (Pearson, 2016). The variation in the molecular responses between males and females identified in Chapters 4 and 5 could help to explain these differences as there was a lot of individual variation in response. This emphasises that the interaction between flight and disease is highly complex, and the response is influenced by pathogenicity and viral load as well as environmental and behavioural factors. Genetic variation is likely to driving these differences in how each individual responds to infection, and this will consequently have effects on populations based on whether these individuals are migratory or resident. To fully understand the impact of disease on migratory insect populations further work will need to be carried out, but this thesis has helped to explain the underlying molecular responses to disease that could drive population structure by altering migratory capacity based on compromises to manage energy demands.

Overall, this research has improved our current understanding of the underlying mechanisms that enable long distance flight in insects. In summary, the stress of flight triggers changes to gene transcription across all areas including reproduction, metabolism, protein production and immunity. Furthermore, strong evidence is provided for the differences between males and females, and how these differences could contribute to improving understanding of how theories such as Bateman's principle and the susceptible male hypothesis can be applied to insects. Considering that we now know that microbial natural enemies are present in field populations of fall armyworm, it is important to consider the impact that these infections could be having on the population ecology and movements of this invasive insect pest and understand the molecular mechanisms underpinning these effects.

6.4 Further work

This thesis has answered many questions about the population structure and ecology of fall armyworm in Africa. In this section, I expand on some of the future work the findings presented here could lead to.

Our results show that microsatellites can be used to successfully determine population structure in invasive fall armyworm, and that they can provide a clearer picture than the traditionally used mitochondrial markers (Withers et al., 2021). Now microsatellites have been used successfully in fall armyworm samples from six African countries, it would be highly valuable to repeat this work on samples from a wider geographic range to further improve understanding of the population structure of invasive fall armyworm. This range should include more sampling sites in Africa as well as the more recently invaded regions including India, China, and Australasia. Furthermore, for this work, I used microsatellites previously designed based on fall armyworm in their native region (Arias et al., 2011; Pavinato, 2013; Arias et al., 2019). However, now genome sequences are available for the invasive fall armyworm population, new microsatellite design is possible that might have more variable regions, or potentially new markers could be developed to resolve the conflict between the dominant hybrid strain (corn/rice) in invasive fall armyworm (Zhang et al., 2020). Considering the identification of insecticide resistance in invasive populations, this work could be vital in helping farmers in Africa, Asia, and Australasia understand the movements of fall armyworm. This would enable them to improve management methods by knowing areas more at risk, and understanding where insecticide resistance may spread should new resistances be identified.

The findings presented here revealed that soon after fall armyworm arrived in Africa it was exposed to a variety of different microbial natural enemies. Within three years it was facing challenges from two viruses (SfMNPV and SpexNPV), a bacterium (*Wolbachia*) and a fungal pathogen (*M. rileyi*). One of the reasons that invasive species can spread rapidly is often thought to be due to the enemy release hypothesis (Cornell and Hawkins, 1993; Torchin and Mitchell, 2004). However, the rapid infection of fall armyworm with pathogens associated with its native range, as well as new pathogens because of species spillover (SpexNPV and *Wolbachia*), suggests that fall armyworm may not have escaped as many enemies as previously thought. This could mean that the rapid spread of fall armyworm is driven by other factors. Thus raising important questions for our understanding of the

population ecology of invasive species, and highlighting the importance of screening for microbial natural enemies in invasive crop pests as soon as possible after invasion.

The impacts of the microbial natural enemies on field populations of fall armyworm need to be determined. This is particularly important for the two pathogens that had not previously been recorded in fall armyworm, SpexNPV and *Wolbachia.* The impacts of these natural enemies on fall armyworm are not yet fully understood, and a bioassay to determine the effects of SpexNPV in fall armyworm was carried out that showed no mortality. However, the SpexNPV strain infecting fall armyworm in the field has not yet been isolated and could have different virulence compared to the strain used in this experiment. Understanding these effects will be vital to fully understand invasive fall armyworm populations, for example, SpexNPV and *Wolbachia* play key roles in the population control of African armyworm*,* including driving haplotype diversity and changing sex ratios through the presence of a male killer strain of *Wolbachia* (Graham and Wilson, 2012; Graham et al., 2012). Whilst the current prevalence of SpexNPV and *Wolbachia* in African fall armyworm is low (0 to 13% and 0 to 4%, respectively, in this study), this could rise over time as population densities of fall armyworm increase, or pathogen evolution could mean that virulence increases. By further work being carried out to fully understand the impact of these pathogens on fall armyworm, it is hoped that their presence could be incorporated into Integrated Pest Management programmes. This could lead to benefits for farmers that are now faced with this invasive crop pest, including reduced insecticide use and higher crop yields.

6.5 Broader Implications

The findings of this thesis have the potential to improve our understanding of invasive fall armyworm populations and how they can be controlled to reduce yield losses caused by this devastating crop pest.

This thesis provides evidence that four different microbial natural enemies are present in fall armyworm in Africa. This could have important implications for biocontrol as the biocontrol agent SfMNPV was found to be present in Ghana, Malawi and Sudan. As SfMNPV is currently in the process of being registered for use in Africa, the knowledge that the virus is already present in wild populations across Africa could support these applications to licence SfMNPV (Bateman et al., 2018; Bateman et al., 2021). Furthermore, understanding the prevalence of natural enemies is essential for effective Integrated Pest Management (IPM) programmes (Ehler, 2006). The findings that SfMNPV, *M. rileyi* and *Wolbachia* are all

present in fall armyworm populations should now be included in IPM approaches in Africa, as the effects of these can be significant in controlling pest populations. For example, in North East India, *M. rileyi* and SfMNPV were responsible for around 50% of fall armyworm mortalities in the field (Mallapur et al., 2018; Souza et al., 2019).

Another implication of this work is the knowledge that microsatellites are more variable in invasive Lepidopteran populations compared to mitochondrial markers. Consequently, microsatellites can detect finer details in population structure compared to the traditional COIB and Tpi approaches (Withers et al., 2021). This could be highly beneficial in the future as understanding how fall armyworm populations move and mix with others can help predict outbreaks as well as warn of insecticide resistances spreading (Zhang et al., 2020; Nagoshi et al., 2017a).

Additionally, the microsatellite approach used in this thesis revealed that fall armyworm were regularly travelling across Africa forming a continental-wide population (Withers et al., 2021). This could have policy implications as working across country borders could greatly improve the response to fall armyworm by helping neighbouring countries, such as warning of potential outbreaks. Additionally, should insecticide resistances be detected in one region or country, warning neighbouring regions of this could reduce crop losses by ensuring farmers know the insecticides most likely to be effective.

Whilst this research will be highly beneficial for the study of fall armyworm populations, it is also transferable to other invasive pest species. This could inform research on populations of other invasive species that might have low genetic diversity due to how invasive species pass through a genetic bottleneck during the early invasion stages. The findings of microbial natural enemies being present in the fall armyworm from when it first arrived suggest that microbial natural enemies should be identified in invasive species as soon as possible after invasions occur. Understanding the prevalence of natural enemies, particularly those which can be used as biocontrol agents, could lead to improved IPM and faster registration of effective biocontrol agents.

Overall, the results presented in this thesis on the identification of natural enemies and improved understanding of fall armyworm populations across Africa will have important implications for policy and biocontrol.

6.6 Summary

This thesis greatly contributes to improving current understanding of fall armyworm in Africa, and how disease could be affecting populations. It will also have wider-reaching benefits and inform researchers of other invasive species, insect immunologists and molecular biologists. The main findings of this thesis are:

- Improved understanding of natural enemies in fall armyworm, and when and where microbial natural enemies may be present at higher levels.
- The enemy release hypothesis may not always be applicable to invasive pest species and their microbial natural enemies. Consequently, invasive crop pests should be screened as soon as possible after arrival to improve understanding of their population ecology, and the role the interactions the pest has with natural enemies could have in pest control.
- Determining population structure is vital in invasive, migratory crop pests, and that this can be achieved by using microsatellites in the fall armyworm as this approach can detect finer genetic differentiation than COI or Tpi markers.
- Males and females respond to infection differently both at the molecular level and behaviourally. Therefore, generalising results to both females and males should be done with caution in entomological research, and whenever possible, both sexes should be included and analysed separately.
- Prior to this research it was thought that the Toll-pathway was predominantly activated in response to bacteria, whereas here results provide evidence that the Toll-pathway was involved in the response to SfMNPV in fall armyworm.

To conclude, by combining laboratory experiments and field collections with genetic analysis this thesis has greatly enhanced our understanding of an invasive crop pest, and the interactions between long distance flight and disease that affect field populations. It is hoped that this work will be continued to ensure that current knowledge of invasive crop pests continues to grow, and that this knowledge will be of great benefit to the farmers and communities most affected by them.

Published Papers

Amy J. Withers, Jolanda de Boer, Gilson Chipabika, Lei Zhang, Judith A. Smith, Christopher M. Jones and Kenneth Wilson. (2021) ***Microsatellites reveal that fall armyworm (Spodoptera frugiperda) populations frequently mix with others throughout Africa.*** *Scientific Reports.* doi.org/10.1038/s41598-021-00298-3

Lei Zhang, Bo Liu, Weigang Zheng, Conghui Liu, Dandan Zhang, Shengyuan Zhao, Zaiyuan Li, Pengjun Xu, Kenneth Wilson, **Amy Withers**, Christopher M. Jones, Judith A. Smith, Gilson Chipabika, Donald L. Kachigamba, Kiwoong Nam, Emmanuelle d'Alençon, Bei Liu, Xinyue Liang, Minghui Jin, Chao Wu, Swapan Chakrabarty, Xianming Yang, Yuying Jiang, Jie Liu, Xiaolin Liu, Weipeng Quan, Guirong Wang, Wei Fan, Wanqiang Qian, Kongming Wu and Yutao Xiao (2020) ***Genetic structure and insecticide resistance characteristics of fall armyworm populations invading China.*** *Molecular Ecology Resources*. 20: 1682– 1696. doi.org/10.1111/1755-0998.13219

Shang Wang, Melissa Minter, Rafael A. Homem, Louise V. Michaelson, Herbert Venthur, Ka S. Lim, **Amy Withers**, Jinghui Xi, Christopher M. Jones and Jing-Jiang Zhou (2020) ***Odorant binding proteins promote flight activity in the migratory insect, Helicoverpa armigera.*** Molecular Ecology Resources. 29: 3795– 3808. doi.org/10.1111/mec.15556

These papers have been attached to the end of this thesis.

Appendices

Appendix A: Supplementary Information for Chapter One

Table S1: Differentially expressed genes in migrating or long-distance flying Lepidoptera.

Gene	Regulation	Function	Species	Comparison between	Method	Reference
turtle	Up	Coordinated motor control	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Brain expressed sequence tag	(Zhu et al., 2008a)
allototropin	Down	Stimulates JH synthesis	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Brain expressed sequence tag	(Zhu et al., 2008a; Zhu et al., 2009)
juvenile hormone (JH) acid methyltransferase	Down	Mediates final step of JH biosynthesis	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Brain expressed sequence tag	(Zhu et al., 2008a; Zhu et al., 2009)
takeout	Down	Potential JH binding protein involved in circadian clock	Monarch butterfly (*Danaus plexippus*)	Wild-caught migrating individuals and non-migratory (summer) individuals	Brain expressed sequence tag	(Zhu et al., 2008a; Zhu et al., 2009)
halipase28	Up	Lipid mobilisation	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
phospholipase A2	Up	Lipid mobilisation	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
acyl-coenzyme A dehydrogenase	Up	Beta-oxidation (Fatty acid metabolism)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
3-hydroxyacyl-coA dehydrogenase	Up	Beta-oxidation (Fatty acid metabolism)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
putative fatty acyl-CoA reductase	Up	Beta-oxidation (Fatty acid metabolism)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
peroxisomal acyl-coezyme A oxidase	Up	Beta-oxidation (Fatty acid	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)

		metabolism)				
apolipophorin	Down (for long distance)	Lipid transport	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
fatty acid synthase (HaOG207601)	Down	Fatty acid synthesis	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
elongase (ELOVL2)	Down	Fatty acid synthesis	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
acyl-CoA delta 11 desaturase	Down	Fatty acid synthesis	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
3-oxoacyl-acyl-carrier-protein reductase	Down	Fatty acid synthesis	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
succinyl-Co-A 3-ketoacid coenzyme A transferase 1	Up	Ketone catabolism (flight fuel)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
inositol-triphosphate 3-kinase B	Up	Lipid signalling (inactivation of fatty acid synthesis)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
cyclic AMP response element-binding protein A	Down (all apart from Anyang short distance fliers)	Lipid signalling	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
GSTs8	Up	Glutathione-s-transferase with antioxidant properties	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
GSTe3	Up	Glutathione-s-transferase with antioxidant properties	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
GSTs3	Down	Glutathione-s-transferase with antioxidant properties	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
OBP6	Up	Odorant binding	Cotton bollworm	Long and short distance fliers	Transcriptomics and	(Jones et al., 2015)

		protein (olfaction, lipid transport)	(*Helicoverpa armigera*)	on tethered flight mills	quantitative PCR	
OBP3	Up	Odorant binding protein (olfaction, lipid transport)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
OBP5	Up	Odorant binding protein (olfaction, lipid transport)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
OBP42	Down	Odorant binding protein (olfaction, lipid transport)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
lysyl oxidase 3-like	Down	Flight muscle structure and function (greater flight efficiency)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
collagen II alpha-1 chain	Down	Flight muscle structure and function (greater flight efficiency)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
myofilin isoform A	Up	Flight muscle structure and function (greater flight efficiency)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
myofilin isoform B	Down	Flight muscle structure and function (greater flight efficiency)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
neuropeptide receptor A16	Down	Prevents JH biosynthesis stimulation	Cotton bollworm	Long and short distance fliers	Transcriptomics and	(Jones et al., 2015)

		(*allototropin* receptor)	(*Helicoverpa armigera*)	on tethered flight mills	quantitative PCR	
neuropeptide receptor B3	Down	Prevents JH biosynthesis stimulation (a*llototropin* receptor)	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
epoxide hydrolase	Up	Degrades JH	Cotton bollworm (*Helicoverpa armigera*)	Long and short distance fliers on tethered flight mills	Transcriptomics and quantitative PCR	(Jones et al., 2015)
actin regulated protein 5	Up	Cytoskeleton organisation	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
CG5045-PA	Up	ATP dependent proteolysis	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
eukaryotic translation initiation factor 3 subunit	Up	Immune response and translation initiation	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
CG6359-PB	Up	Immune response	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
CG9300-PA	Up	Transport	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a	(Zhu et al., 2009)

					brain expressed sequence tag	
CG32687-PA	Up	Protein binding	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
cytochrome P450 18a1	Down	Oxidative metabolism (heme binding, monoxygenase activity, electron carrier)	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
HMG coenzyme A synthase	Down	Isoprenoid biosynthetic activity	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
CG1317-PB	Down	Protein / zinc ion binding	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
patj	Down	Apical protein localization, cell-cell junction assembly, morphogenesis of follicular epithelium	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
CDP diglyceride synthetase	Down	Lipid metabolism, CDP-	Monarch butterfly	Migrating individuals and non-migratory	Microarray analysis of cDNA	(Zhu et al., 2009)

		diaglycerol biosynthesis, phototransduction	(*Danaus plexippus*)	(summer) individuals	sequences obtained using a brain expressed sequence tag	
tyramine β hydroxylase	Down	Larval locomotory behaviour, memory, motor behaviour, ovulation	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
capa receptor	Down	G-protein coupled receptor, neuropeptide receptor	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
CG8032-PA	Down	Electron carrier activity, oxidoreductase	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
vrille	Down	Circadian rhythm (negative regulator of *clock*), locomotor rhythm, bristle morphogenesis, transcription regulator activity	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
CG4250-PA	Down	G-protein signalling, intracellular signalling, proteolysis	Monarch butterfly (*Danaus plexipus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain	(Zhu et al., 2009)

					expressed sequence tag	
CG7379-PA	Down	Protein / zinc ion binding, apoptosis	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
growl	Down	Folic acid biosynthesis, folinic acid breakdown	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
abrupt	Down	Axon choice point recognition, neuron development, sec determination	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
CG31140-PB	Down	Activation of protein kinase C, diacylglycerol kinase activity	Monarch butterfly (*Danaus plexipus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
CG31717-PA	Down	Catalytic activity	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
CG33936-PA	Down	DNA binding, zinc ion binding	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory	Microarray analysis of cDNA sequences	(Zhu et al., 2009)

				(summer) individuals	obtained using a brain expressed sequence tag	
will die slowly	Down	Cell proliferation	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
synapse associated protein 47kD	Down	Synaptic transmission	Monarch butterfly (*Danaus plexippus*)	Migrating individuals and non-migratory (summer) individuals	Microarray analysis of cDNA sequences obtained using a brain expressed sequence tag	(Zhu et al., 2009)
Cryptochrome	Up	Diretionality	Black cutworm (*Agrotis ipsilon*)	Lab-reared and migratory individuals	RT-PCR	(Chang et al., 2019)
Magnetoreceptor	Up	Directionality	Black cutworm (*Agrotis ipsilon*)	Lab-reared and migratory individuals	RT-PCR	(Chang et al., 2019)

Appendix B: Supplementary Information for Chapter Two

The species specificity of the two primers designed for SfMNPV and SpexNPV was tested using three groups of DNA samples (Table S2, Fig. S1). This showed that the primers designed for SpexNPV did not amplify any DNA in samples with SfMNPV and that the SfMNPV primers did not amplify any DNA in samples with SpexNPV (Table S2, Fig. S1). There was a band visible in two samples using the SfMNPV primers known SpexNPV infection (Fig. S1 B), however, this band was much smaller than the expected band for the SfMNPV primers so could be dismissed.

Table S2: Species specificity of SfMNPV and SpexNPV primers used in this study.

Group	**Block code in Figure A1**	**SpexNPV primers (Orf 57-58)**	**SfMNPV primers (sfpg 41.1)**
SpexNPV DNA extracted from *Spodoptera exempta* with known SpexNPV virus (N=3, each sample repeated twice)	A	Amplified	No amplification
		Amplified	No amplification
		Amplified	No amplification
		Amplified	No amplification
		Amplified	No amplification (visible band wrong size)
		Amplified	No amplification (visible band wrong size)
SfMNPV DNA extracted from SfMNPV virus (AgBiTech) (N=3, each sample repeated twice)	B	No amplification	Amplified
		No amplification	Amplified
		No amplification	Amplified
		No amplification	Amplified
		No amplification	No amplification
		No amplification	No amplification
SfMNPV DNA from *Spodoptera frugiperda* with known SfMNPV (N=3, each sample repeated twice)	C	No amplification	Amplified
		No amplification	Amplified
		No amplification	Amplified
		No amplification	Amplified
		No amplification	Amplified
		No amplification	Amplified

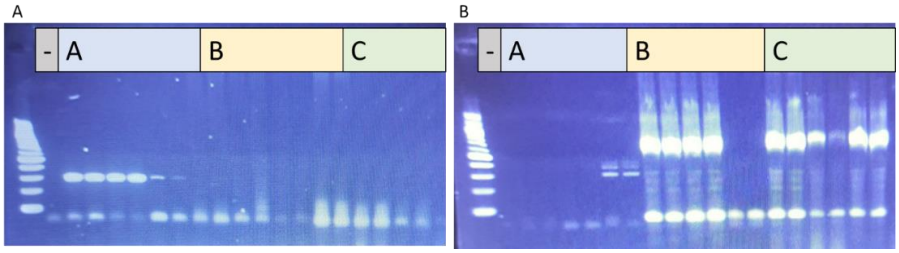


Figure S1: Species specificity of SfMNPV and SpexNPV primers. A) SpexNPV primers amplified with known SpexNPV infection (block A), SfMNPV viral DNA (block B) and SfMNPV infection (block C) and B) SfMNPV primers amplified with known SpexNPV infection (block A), SfMNPV viral DNA (block B) and SfMNPV infection (block C).

Table S3: Ingredients for the artificial diet used for *Spodoptera frugiperda* larvae. Once produced and set into individual small polypots, diet was stored at 4°C until needed.

Ingredients	**Amount for approximately 200 pots (g)**
Wheat germ	72
Casein	33
Sugar	29.25
Yeast	14.25
Wesson's salt	9.37
Sorbic acid	1.5
Cholesterol	0.93
P-hydroxybenzoic acid	0.93
Linseed oil*	1.87
Distilled water	862.5 ml
Agar powder (2%)	18.75 g
Vitamin mix (Nicotinic acid, pantothenic acid, riboflavin, thiamine, pyridoxine, folic acid, D-Biotin)	0.093
Ampicillin mix* (from premixed formula composed of 25g ampicillin and 100g L-Ascorbic acid)	5.53
Kanamycin mix* (from premixed formula composed of 13.95g kanamycin and 111.05g L-Ascorbic acid)	4.98
Choline	0.93

* Antibiotics were alternated to reduce the risk of resistance developing.

Table S3: Larvae samples collected in Zambia showing overt signs of fungal infection. Universal fungal primers designed to amplify EF1 were used (EF1-1002F: 5'- TTCATCAAGAACATGAT -3'; EF1-1688R: 5'- GCTATCATCACAATGGACGTTCTTGAAG -3') (Stielow et al., 2015). The sequence amplified for each sample are shown in the table below.

Sample	**Sequence amplified**
	No amplification
	Mixed sequence
	CACTCTGCTTGCCTACACCTTGGGTGTCAAGCAGCTCATCGTTGCCATCAACAAGATGGATA CCACCAAGTGGTCTGAGGCCCGTTACCAGGAGATCATCAAGGAGACGTCCAACTTCATCAA GAAGGTCGGCTACAACCCCAAGACTGTTGCCTTCGTCCCCATCTCCGGCTTCCACGGAGACA ACATGCTTCAGGCCTCGACCAACTGCCCCTGGTACAAGGGCTGGGAGAAGGAGACCAAGG CTGGCAAGTCCACTGGCAAGACCCTTCTTGAGGCCATTGACGCCATTGAGCCCCCCAAGCGT CCCACCGACAAGCCCCTGCGTCTTCCCCTCCAGGATGTCTACAAGATCGGTGGTATTGGAAC TGTCCCTGTCGGCCGTATCGAGACTGGTATCATCAAGCCTGGTATGGTCGTTACCTTCGCCC CCTCCAACGTTACCACTGAAGTCAAGTCTGTGGAAATGCACCACGAGCAGCTTTCTGAGGGT GTTCCCGGTGACAACGTTGGTTTCAATGTGAAGAACGTTTCTGTCAAGGAAA
	CTGCTTGCCTACACCTTGGGTGTCAAGCagCTCATCGTTGCCATCAACAAGATGGATACCACC AAGTGGTCTGAGGCCCGTTACCAGGAGATCATCAAGGAGACGTCCAACTTCATCAAGAAGG TCGGCTACAACCCCAAGACTGTTGCCTTCGTCCCCATCTCCGGCTTCCACGGAGACAACATG CTTCAGGCCTCGACCAACTGCCCCTGGTACAAGGGCTGGGAGAAGGAGACcAAGGcTGGCA AGTCCACTGGCAAGACCCTTCTTGAGGCCATTGACGCCATTGAGCCCCCCAAGCGTCCCACC GACAAGCCCCTGCGTCTTCCCCTCCAGGATGTCTACAAGATCGGTGGTATTGGAACTGTCCC TGTCGGCCGTATCGAGACTGGTATCATCAAGCCTGGTATGGTCGTTACCTTCGCCCCCTCCA

	ACGTTACCACTGAAGTCAAGTCTGTGGAAATGCACCACGAGCAGCTTTCTGAGGGTGTTCCC GGTGACAACGTTGGTTTCAATGTGAAGAACGTTTCTGTCAAGGAAA
 	CTCTGCTTGCCTACACCTTGGGTGTCAAGCAGCTCATCGTTGCCATCAACAAGATGGATACC ACCAAGTGGTCTGAGGCCCGTTACCAGGAGATCATCAAGGAGACGTCCAACTTCATCAAGA AGGTCGGCTACAACCCCAAGACTGTTGCCTTCGTCCCCATCTCCGGCTTCCACGGAGACAAC ATGCTTCAGGCCTCGACCAACTGCCCCTGGTACAAGGGCTGGGAGAAGGAGACCAAGGCT GGCAAGTCCACTGGCAAGACCCTTCTTGAGGCCATTGACGCCATTGAGCCCCCCAAGCGTCC CACCGACAAGCCCCTGCGTCTTCCCCTCCAGGATGTCTACAAGATCGGTGGTATTGGAACTG TCCCTGTCGGCCGTATCGAGACTGGTATCATCAAGCCTGGTATGGTCGTTACCTTCGCCCCC TCCAACGTTACCACTGAAGTCAAGTCTGTGGAAATGCACCACGAGCAGCTTTCTGAGGGTG TTCCCGGTGACAACGTTGGTTTCAATGTGAAGAACGTTTCTGTCAAGGAAATCCGCCGTGGT AACGTTGCC
 	Mixed sequence
 	Mixed sequence

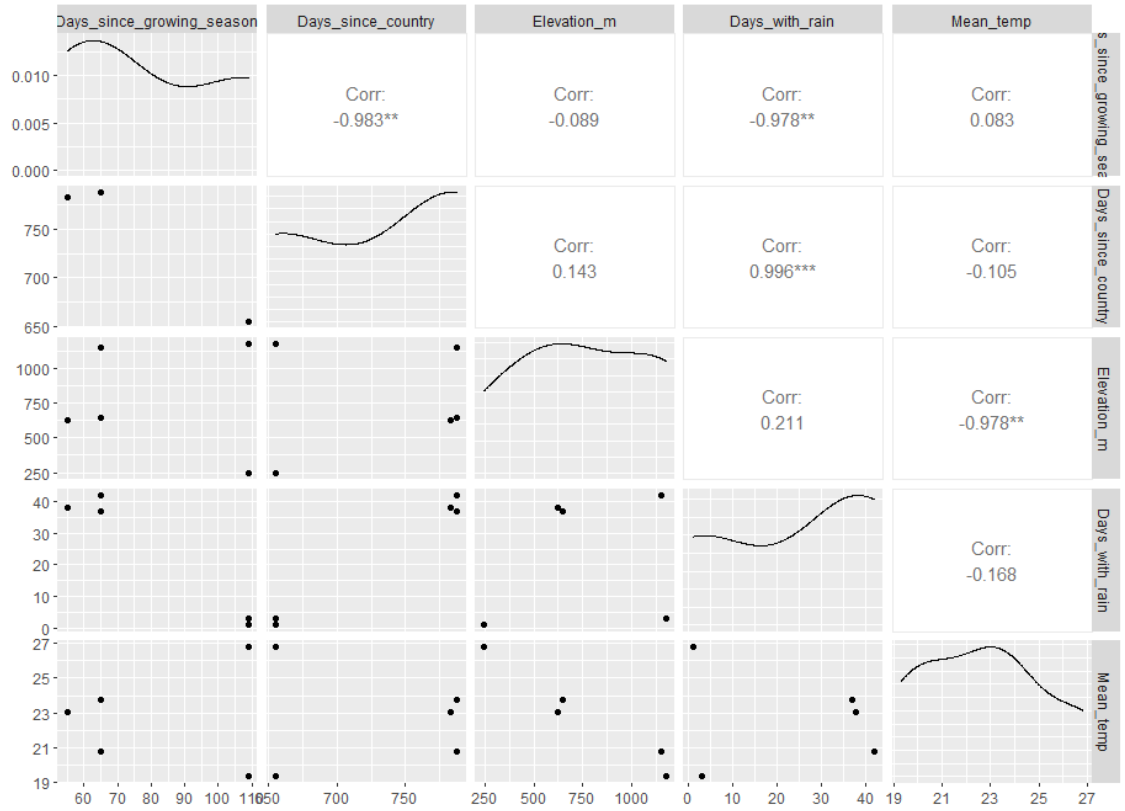


Figure S2: A comparison of the variables showing strong correlation between some variables in Malawi. Significance is shown with asterisk where *** indicates *P<0.001,* ** indicates *P<0.01* and * indicates *P<.0.05*.

Appendix C: Supplementary Information for Chapter Three

Figure S3: The migratory route of the African armyworm (*S. exempta).* Based on microsatellite clustering patterns we hypothesise that the fall armyworm moves in a similar pattern around Africa following the prevailing winds of the inter-tropical convergence zone. (Image from Chapman *et al.* 2015, based on Rose *et al.* 2000).

Appendix D: Supplementary Information for Chapter Four

Introduction

The cotton bollworm (Helicoverpa armigera) is a highly invasive crop pest, native to Europe, Asia, and Africa, it is invasive in South America and the Caribbean. Similarly to the fall armyworm, it undertakes long distance migratory flights, which that been a key factor in its rapid spread (Jones et al., 2019).

Methods

Twenty-four female H. armigera that had not been exposed to any nucleopolyhedroviruses were attached to flight mills (Fig. S4) and left to fly overnight. The following morning RNA was extracted and sequenced. Differential gene expression analysis was then carried out. RNA extraction and differential expression analysis was all carried out using the same protocol as outlined for FAW in this chapter.

Results and Discussion

Interestingly, many components of the Toll-pathway were upregulated in response to flight in H. armigera that had not been exposed to an immune challenge; clip-domain serine proteases (e.g., Easter), Toll-like receptor 6, pelle, cactus, defensin and cecropin B (Fig. S4). This upregulation of Toll-pathway components was observed in FAW following flight, suggesting that the Toll-pathway may be activated in response flight in multiple Lepidoptera species. Similarly to FAW, numerous other genes with a potential role in immunity were upregulated during flight, such as Hdd genes (Hdd1m Hdd13, Hdd23 and Hdd11) (Fig. S4). As the H. armigera in this experiment were not infected, this suggests that the upregulation of immune genes may be triggered by the general stress of flight.

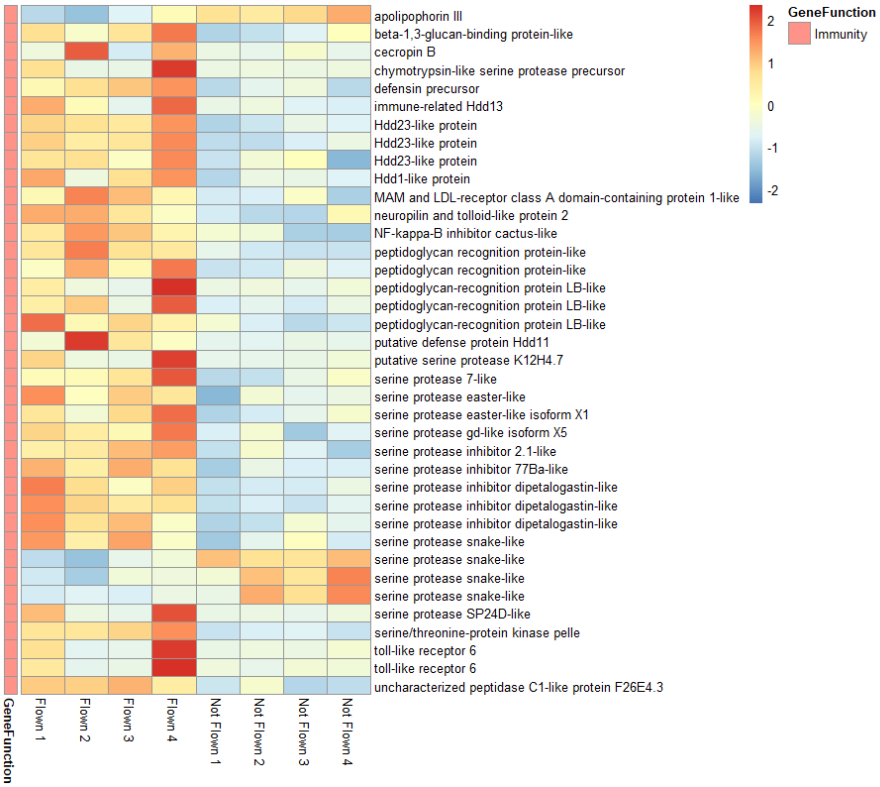


Fig. S4: A heatmap showing the differentially expressed genes with a proposed function in immunity in *H. armigera* females during flight. Blue show downregulated genes and red show upregulated genes.

Appendix E: Supplementary Information for Chapter Five

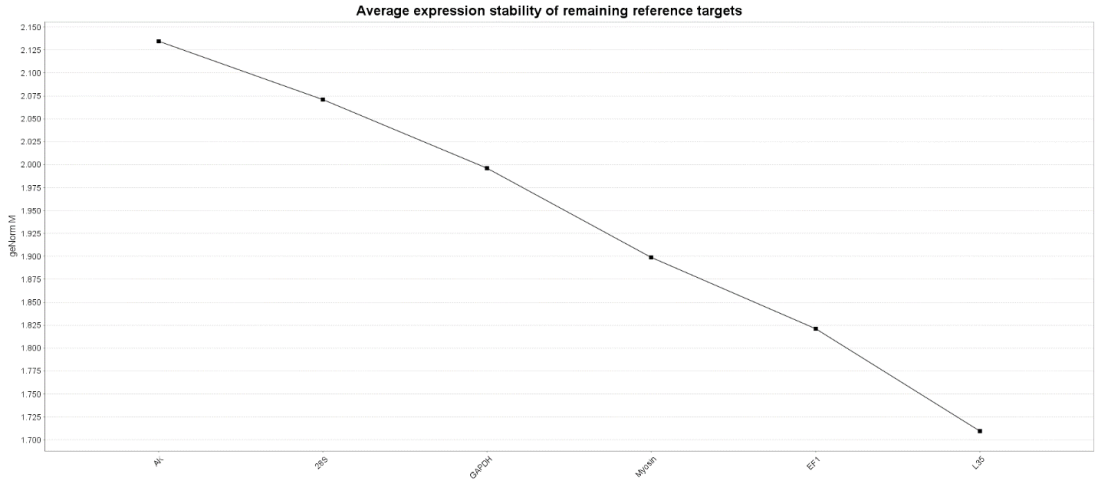


Figure S5: The average expression stability value for the six trialled reference genes. This was calculated using the GeNorm M method on the qbase+ software.

Table S4: The results of the principal components analysis (PCA) on the 16 flight variables. The principal components (PC) with an eigenvalue >1 are shaded in grey. The variables with the greatest contributions to each PC (>0.3 or <-0.3) are shown in bold.

	Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13	PC14	PC15	PC16
Distance	Total distance	**0.33**	-0.04	0.03	-0.08	-0.20	0.37	-0.28	-0.17	0.35	-0.20	-0.27	**-0.41**	**-0.34**	0.28	-0.06	-0.03
	Average flight distance	0.29	-0.05	**-0.38**	0.19	-0.07	0.03	0.14	**0.35**	0.17	0.06	-0.16	0.26	-0.25	**-0.32**	**-0.53**	0.06
	First flight distance	0.19	-0.23	**0.48**	0.24	0.18	0.16	0.02	0.22	0.01	0.10	-0.06	0.29	**-0.41**	-0.16	**0.46**	0.01
	Furthest flight distance	**0.32**	-0.22	-0.08	0.01	0.08	-0.18	0.10	-0.15	-0.11	-0.03	-0.11	**-0.31**	0.15	-0.23	0.17	0.74
	Longest flight distance	**0.32**	-0.22	-0.08	0.01	0.08	-0.18	0.11	-0.16	-0.11	-0.02	-0.11	**-0.31**	0.15	**-0.41**	0.12	**-0.66**
Duration	Total duration	0.28	-0.22	0.02	**-0.33**	-0.20	0.29	-0.28	-0.18	0.20	0.02	**0.33**	**0.43**	**0.38**	-0.22	0.02	0.03
	Average flight duration	0.29	-0.06	**-0.41**	0.15	-0.06	-0.04	0.13	**0.30**	0.12	0.03	-0.11	0.14	**0.31**	**0.52**	**0.43**	-0.08
	First flight duration	0.17	-0.27	**0.50**	0.21	0.21	0.06	0.03	0.14	-0.07	0.05	-0.08	-0.07	**0.43**	**0.30**	**-0.49**	-0.02
	Longest flight duration	0.29	-0.26	-0.11	-0.13	0.14	-0.28	0.09	**-0.34**	**-0.30**	-0.08	**0.33**	0.23	**-0.41**	**0.39**	-0.16	-0.04
Speed	Max speed	0.24	**0.34**	0.09	-0.26	-0.08	-0.05	-0.29	0.16	**-0.55**	**-0.31**	**-0.43**	0.24	0.06	-0.02	0.00	0.00
	Average flight speed	0.20	**0.30**	-0.14	**0.34**	-0.01	**0.58**	0.08	-0.09	**-0.46**	0.21	**0.31**	-0.16	0.02	-0.02	0.00	0.00
	First flight mean speed	0.17	**0.34**	0.04	**-0.40**	**0.41**	0.09	0.28	-0.23	0.18	**0.50**	-0.28	0.11	0.01	0.06	-0.02	0.00
	First flight max speed	0.24	**0.30**	0.13	**-0.30**	0.25	-0.03	0.19	**0.44**	0.18	**-0.37**	**0.48**	-0.23	-0.02	-0.05	0.01	0.00
	Longest flight speed	0.15	**0.32**	0.28	0.27	**-0.41**	-0.12	**0.49**	**-0.36**	0.19	-0.29	-0.04	0.21	0.08	-0.01	0.00	0.00
	Longest flight max speed	0.25	0.21	0.20	0.02	**-0.43**	**-0.43**	-0.27	0.16	0.02	**0.56**	0.19	-0.17	-0.07	0.04	-0.01	0.00
No.	Number of flights	-0.12	**-0.32**	0.09	**-0.45**	**-0.46**	0.22	**0.50**	0.23	-0.25	0.12	-0.07	-0.12	-0.07	0.05	0.00	-0.01
Eigenvalue		7.67	3.18	1.78	1.29	0.79	0.52	0.33	0.17	0.13	0.08	0.04	0.02	0.01	0.00	0.00	0.00
Standard deviation		2.77	1.78	1.33	1.13	0.89	0.72	0.57	0.41	0.36	0.28	0.21	0.12	0.10	0.01	0.01	0.00
Variance explained (%)		0.48	0.20	0.11	0.08	0.05	0.03	0.02	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cumulative variation explained (%)		0.48	0.68	0.79	0.87	0.92	0.95	0.97	0.98	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S5: The output of each manova using PC1, PC1 to 2, PC1 to 3 and PC1 to 4, the AIC of each model and the anova comparison of each model to the model using all four PC.

							Comparison to model 4			
Model	**Variable**	**Df**	**Pillai**	**F**	**Pr(>F)**	**AIC**	**Df**	**Pillai**	**F**	**Pr (>F)**
1) manova with PC1	PC1	1	0.51	7.68	**<0.001**	259	40	2.37	3.22	**<0.001**
	Treatment group	1	0.09	0.70	0.597					
	Sex	1	0.21	1.95	0.128					
	PC1: Treatment group	1	0.62	11.86	**<0.001**					
	PC1: Sex	1	0.73	19.34	**<0.001**					
	Treatment group: Sex	1	0.47	6.30	**0.001**					
	PC1: Treatment group: Sex	1	0.26	2.61	0.056					
2) manova with PC1 + PC2	PC1 to 2	2	1.12	8.25	**<0.001**	210	88	2.00	3.68	**<0.001**
	Treatment group	1	0.32	2.94	**0.040**					
	Sex	1	0.20	1.56	0.215					
	PC1 to 2: Treatment group	2	0.62	2.92	**0.009**					
	PC1 to 2: Sex	2	0.89	5.22	**<0.001**					
	Treatment group: Sex	1	0.47	5.48	**0.003**					
	PC1 to 2: Treatment group: Sex	2	0.43	1.77	0.103					
3) manova with PC1 + PC2 + PC3	PC1 to 3	3	1.85	9.59	**<0.001**	65	63	1.39	4.53	**<0.001**
	Treatment group	1	0.65	10.02	**<0.001**					
	Sex	1	0.71	13.45	**<0.001**					
	PC1 to 3: Treatment group	3	1.48	5.83	**<0.001**					
	PC1 to 3: Sex	3	1.36	4.97	**<0.001**					
	Treatment group: Sex	1	0.62	9.13	**<0.001**					
	PC1 to 3: Treatment group: Sex	2	1.07	6.59	**<0.001**					
4) manova with PC1+ PC2 + PC3+ PC4	PC1 to 4	4	2.32	7.61	**<0.001**	25	Not Applicable			
	Treatment group	1	0.91	49.47	**<0.001**					
	Sex	1	0.58	6.54	**0.002**					
	PC1 to 4: Treatment group	4	2.00	5.48	**<0.001**					
	PC1 to 4: Sex	4	1.90	4.96	**<0.001**					
	Treatment group: Sex	1	0.54	5.50	**0.004**					
	PC1 to 4: Treatment group: Sex	2	1.36	10.67	**0.001**					

Abbreviations

- AIC: Akaike information criteria
- AMOVA: Analysis of molecular variance
- Bt: *Bacillus thuringiensis*
- COIB: *Mitochondrial cytochrome oxidase I*
- DAPC: Discriminant Analysis Principal Components
- DNA: Deoxyribonucleic acid
- FAW: Fall armyworm (*Spodoptera frugiperda*)
- GLM: Generalized linear model
- IPM: Integrated pest management
- JH: Juvenile hormone
- MANOVA: Multivariate analysis of variance
- NPV: Nucleopolyhedroviruses
- PCA: Principal components analysis
- PCR: Polymerase chain reaction
- RNA: Ribonucleic acid
- SfMNPV: *Spodoptera frugiperda multiple nucleopolyhedrovirus*
- SpexNPV: *Spodoptera exempta nucleopolyhedrovirus*
- Tpi: *Triosephosphate isomerase*

References

Abe, T., Hemmi, H., Miyamoto, H., Moriishi, K., Tamura, S., Takaku, H., Akira, S. & Matsuura, Y. 2005. Involvement of the Toll-Like Receptor 9 Signaling Pathway in the Induction of Innate Immunity by Baculovirus. *The Journal of Virology,* 79(5)**,** 2847.

Abrahams, P., Bateman, M., Beale, T., Clottey, V., Cock, M., Colmenarez, Y., Corniani, N., Day, R., Early, R., Godwin, J., Gomez, J., Moreno, P. G., Murpy, S. T., Oppong-Mensah, B., Phiri, N., Pratt, C., Richards, G., Silvestri, S. & Witt, A. 2017. Fall Armyworm: Impacts and Implications for Africa. Evidence Note 2: September 2017.

Adamack, A. & Gruber, B. 2014. Popgenreport: Simplifying Basic Population Genetic Analyses in R. *Methods in Ecology and Evolution,* 5.

Adamo, S. A. & Parsons, N. M. 2006. The Emergency Life-History Stage and Immunity in the Cricket, Gryllus Texensis. *Animal Behaviour,* 72(1)**,** 235-244.

Agapow, P.-M. & Burt, A. 2001. Indices of Multilocus Linkage Disequilibrium. *Molecular Ecology Notes,* 1(1-2)**,** 101-102.

Agboyi, L., Goergen, G., Beseh, P., Mensah, S., Clottey, V., Glikpo, R., Buddie, A., Cafa, G., Offord, L., Day, R., Rwomushana, I. & Kenis, M. 2020. Parasitoid Complex of Fall Armyworm, Spodoptera Frugiperda, in Ghana and Benin. *Insects,* 11**,** 68.

Agrawal, A. A. 2000. Mechanisms, Ecological Consequences and Agricultural Implications of Tri-Trophic Interactions. *Current Opinion in Plant Biology,* 3(4)**,** 329-335.

Ahmed, M. Z., Araujo-Jnr, E. V., Welch, J. J. & Kawahara, A. Y. 2015a. Wolbachia in Butterflies and Moths: Geographic Structure in Infection Frequency. *Frontiers in Zoology,* 12(1)**,** 16.

Ahmed, M. Z., Li, S.-J., Xue, X., Yin, X.-J., Ren, S.-X., Jiggins, F. M., Greeff, J. M. & Qiu, B.-L. 2015b. The Intracellular Bacterium Wolbachia Uses Parasitoid Wasps as Phoretic Vectors for Efficient Horizontal Transmission. *PLoS pathogens,* 10(2)**,** e1004672-e1004672.

Alaux, C., Crauser, D., Pioz, M., Saulnier, C. & Le Conte, Y. 2014. Parasitic and Immune Modulation of Flight Activity in Honey Bees Tracked with Optical Counters. *The Journal of Experimental Biology,* 217(19)**,** 3416.

Albrectsen, B. & Nachman, G. 2001. Female-Biased Density-Dependent Dispersal of a Tephritid Fly in a Fragmented Habitat and Its Implications for Population Regulation. *Oikos,* 94(2)**,** 263-272.

Ali, M. I., Felton, G. W., Meade, T. & Young, S. Y. 1998. Influence of Interspecific and Intraspecific Host Plant Variation on the Susceptibility of Heliothines to a Baculovirus. *Biological Control,* 12(1)**,** 42-49.

Alkhatib, S. G. & Landry, J. W. 2011. The Nucleosome Remodeling Factor. *FEBS letters,* 585(20)**,** 3197-3207.

Almarinez, B. J. M., Barrion, A. T., Navasero, M. V., Navasero, M. M., Cayabyab, B. F., Carandang, J. S. R., Legaspi, J. C., Watanabe, K. & Amalin, D. M. 2020. Biological Control: A Major Component of the Pest Management Program for the Invasive Coconut Scale Insect, Aspidiotus Rigidus Reyne, in the Philippines. *Insects,* 11(11).

Altizer, S., Bartel, R. & Han, B. A. 2011. Animal Migration and Infectious Disease Risk. *Science,* 331(6015)**,** 296-302.

Altizer, S. M., Oberhauser, K. S. & Brower, L. 2000. Associations between Host Migration and the Prevalence of a Protozoan Parasite in Natural Populations of Adult Monarch Butterflies. *Ecol. Entomol.,* 25(2)**,** 125-139.

Álvarez, S. P., Guerrero, A. M., Duarte, B. N. D., Tapia, M. A. M., Medina, J. A. C. & Domínguez Rodríguez, Y. 2018. First Report of a New Isolate of Metarhizium Rileyi from Maize Fields of Quivicán, Cuba. *Indian Journal of Microbiology,* 58(2)**,** 222-226.

Alyokhin, A., Drummond, F. A., Sewell, G. & Storch, R. H. 2011. Differential Effects of Weather and Natural Enemies on Coexisting Aphid Populations. *Environmental Entomology,* 40(3)**,** 570-580.

Anderson, C. J., Tay, W. T., Mcgaughran, A., Gordon, K. & Walsh, T. K. 2016. Population Structure and Gene Flow in the Global Pest, Helicoverpa Armigera. *Molecular Ecology,* 25(21)**,** 5296-5311.

Anderson, K. L., Deveson, T. E., Sallam, N. & Congdon, B. C. 2010. Wind-Assisted Migration Potential of the Island Sugarcane Planthopper Eumetopina Flavipes (Hemiptera: Delphacidae): Implications for Managing Incursions across an Australian Quarantine Frontline. *Journal of Applied Ecology,* 47(6)**,** 1310-1319.

Andersson, E. & Isgren, E. 2021. Gambling in the Garden: Pesticide Use and Risk Exposure in Ugandan Smallholder Farming. *Journal of Rural Studies,* 82**,** 76-86.

Andersson, M. 1986. Evolution of Condition-Dependent Sex Ornaments and Mating Preferences: Sexual Selection Based on Viability Differences. *Evolution,* 40(4)**,** 804-816.

Andrews, S. 2014. *Fastqc a Quality Control Tool for High Throughput Sequence Data*.

Apvma 2021. Permit Number - Per90820, Product for Integrated Management of Fall Armyworm in Various Crops.

Arcgis 2021. World_Topo_Map. *In:* ESRI (ed.).

Ardia, D. R., Gantz, J. E., , B. C., Schneider & Strebel, S. 2012. Costs of Immunity in Insects: An Induced Immune Response Increases Metabolic Rate and Decreases Antimicrobial Activity. *Functional Ecology,* 26(3)**,** 732-739.

Arias, O., Cordeiro, E., Corrêa, A. S., Domingues, F. A., Guidolin, A. S. & Omoto, C. 2019. Population Genetic Structure and Demographic History of Spodoptera Frugiperda (Lepidoptera: Noctuidae): Implications for Insect Resistance Management Programs. *Pest Management Science,* 75(11)**,** 2948-2957.

Arias, R. S., Blanco, C. A., Portilla, M., Snodgrass, G. L. & Scheffler, B. E. 2011. First Microsatellites from Spodoptera Frugiperda (Lepidoptera: Noctuidae) and Their Potential Use for Population Genetics. *Annals of the Entomological Society of America,* 104(3)**,** 576-587.

Armenta, R., Martinez, A. M., Chapman, J. W., Magallanes, R., Goulson, D., Caballero, P., Cave, R. D., Cisneros, J., Valle, J., Castillejos, V., Penagos, D. I., Garcia, L. F. & Williams, T. 2003. Impact of a Nucleopolyhedrovirus Bioinsecticide and Selected Synthetic Insecticides on the Abundance of Insect Natural Enemies on Maize in Southern Mexico. *Journal of Economic Entomology,* 96(3)**,** 649-661.

Armes, N. J., Jadhav, D. R. & Desouza, K. R. 1996. A Survey of Insecticide Resistance in Helicoverpa Armigera in the Indian Subcontinent. *Bulletin of Entomological Research,* 86(5)**,** 499-514.

Arrese, E. L. & Soulages, J. L. 2010. Insect Fat Body: Energy, Metabolism, and Regulation. *Annu. Rev. Entomol.,* 55**,** 207-225.

Arrizubieta, M., Williams, T., Caballero, P. & Simón, O. 2014. Selection of a Nucleopolyhedrovirus Isolate from Helicoverpa Armigera as the Basis for a Biological Insecticide. *Pest Management Science,* 70(6)**,** 967-976.

Asser-Kaiser, S., Radtke, P., El-Salamouny, S., Winstanley, D. & Jehle, J. A. 2011. Baculovirus Resistance in Codling Moth (Cydia Pomonella L.) Caused by Early Block of Virus Replication. *Virology,* 410(2)**,** 360-367.

Avadhanula, V., Weasner, B., Hardy, G., Kumar, J. & Hardy, R. 2009. A Novel System for the Launch of Alphavirus Rna Synthesis Reveals a Role for the Imd Pathway in Arthropod Antiviral Response. *PLoS pathogens,* 5**,** e1000582.

Badenes-Perez, F. R. & Shelton, A. M. 2006. Pest Management and Other Agricultural Practices among Farmers Growing Cruciferous Vegetables in the Central and

Western Highlands of Kenya and the Western Himalayas of India. *International Journal of Pest Management,* 52(4)**,** 303-315.
Barcellos-Hoff, M. H. & Dix, T. A. 1996. Redox-Mediated Activation of Latent Transforming Growth Factor-Beta 1. *Molecular Endocrinology,* 10(9)**,** 1077-1083.
Barrera, G. P., Belaich, M. N., Patarroyo, M. A., Villamizar, L. F. & Ghiringhelli, P. D. 2015. Evidence of Recent Interspecies Horizontal Gene Transfer Regarding Nucleopolyhedrovirus Infection of Spodoptera Frugiperda. *Bmc Genomics,* 16.
Bartel, R. A., Oberhauser, K. S., Roode, J. C. D. & Altizer, S. M. 2011. Monarch Butterfly Migration and Parasite Transmission in Eastern North America. *Ecology,* 92(2)**,** 342-351.
Barthel, A., Staudacher, H., Schmaltz, A., Heckel, D. G. & Groot, A. T. 2015. Sex-Specific Consequences of an Induced Immune Response on Reproduction in a Moth. *BMC evolutionary biology,* 15**,** 282-282.
Bartoń, K. 2013. *Mumin: Multi-Model Inference*.
Bateman, A. J. 1948. Intra-Sexual Selection in Drosophila. *Heredity,* 2(3)**,** 349-368.
Bateman, M. L., Day, R. K., Luke, B., Edgington, S., Kuhlmann, U. & Cock, M. J. W. 2018. Assessment of Potential Biopesticide Options for Managing Fall Armyworm (Spodoptera Frugiperda) in Africa. *Journal of Applied Entomology,* 142(9)**,** 805-819.
Bateman, M. L., Day, R. K., Rwomushana, I., Subramanian, S., Wilson, K., Babendreier, D., Luke, B. & Edgington, S. 2021. Updated Assessment of Potential Biopesticide Options for Managing Fall Armyworm (Spodoptera Frugiperda) in Africa. *Journal of Applied Entomology,* 145(n/a)**,** 384-393.
Beenakkers, A. M. T., Van Der Horst, D. J. & Marrewijk, W. J. A. 1984. Insect Flight Muscle Metabolism. *Insect Biochemistry and Molecular Biology,* 14**,** 243-260.
Behle, R. W. & Popham, H. J. R. 2012. Laboratory and Field Evaluations of the Efficacy of a Fast-Killing Baculovirus Isolate from Spodoptera Frugiperda. *Journal of Invertebrate Pathology,* 109(2)**,** 194-200.
Bellard, C., Cassey, P. & Blackburn, T. M. 2016. Alien Species as a Driver of Recent Extinctions. *Biology letters,* 12(2)**,** 20150623-20150623.
Belmonte, R. L., Corbally, M.-K., Duneau, D. F. & Regan, J. C. 2020. Sexual Dimorphisms in Innate Immunity and Responses to Infection in Drosophila Melanogaster. *Frontiers in immunology,* 10**,** 3075-3075.
Belvin, M. P. & Anderson, K. V. 1995. Cactus Protein Degradation Mediates Drosophila Dorsal-Ventral Signaling. *Genes Dev.,* 9(7)**,** 783-793.
Bennett, K. L., Gómez Martínez, C., Almanza, A., Rovira, J. R., Mcmillan, W. O., Enriquez, V., Barraza, E., Diaz, M., Sanchez-Galan, J. E., Whiteman, A., Gittens, R. A. & Loaiza, J. R. 2019. High Infestation of Invasive Aedes Mosquitoes in Used Tires Along the Local Transport Network of Panama. *Parasites & Vectors,* 12(1)**,** 264.
Berwaerts, K., Van Dyck, H. & Aerts, P. 2002. Does Flight Morphology Relate to Flight Performance? An Experimental Test with the Butterfly Pararge Aegeria. *Functional Ecology,* 16(4)**,** 484-491.
Black, J. L., Clark, M. K. & Sword, G. A. 2022. Physiological and Transcriptional Immune Responses of a Non-Model Arthropod to Infection with Different Entomopathogenic Groups. *PLOS ONE,* 17(2)**,** e0263620.
Blackmer, J. L., Naranjo, S. E. & Williams, L. H., Iii 2004. Tethered and Untethered Flight by Lygus Hesperus and Lygus Lineolaris (Heteroptera: Miridae). *Environmental Entomology,* 33(5)**,** 1389-1400.
Boaventura, D., Martin, M., Pozzebon, A., Mota-Sanchez, D. & Nauen, R. 2020. Monitoring of Target-Site Mutations Conferring Insecticide Resistance in Spodoptera Frugiperda. *Insects,* 11(8).
Boogaard, H. & Grijn, G. V. D. 2021. Agrometerological Indicators from 1997 to Present Derived from Reanalysis: Precipitation Flux. 2020-01-30 ed.

Bradley, C. & Altizer, S. 2005. Parasites Hinder Monarch Butterfly Flight: Implications for Disease Spread in Migratory Hosts. *Ecol. Lett.,* 8(3)**,** 290-300.

Bradshaw, C. J. A., Leroy, B., Bellard, C., Roiz, D., Albert, C., Fournier, A., Barbet-Massin, M., Salles, J.-M., Simard, F. & Courchamp, F. 2016. Massive yet Grossly Underestimated Global Costs of Invasive Insects. *Nature Communications,* 7(1)**,** 12986.

Brenton-Rule, E. C., Barbieri, R. F. & Lester, P. J. 2016. Corruption, Development and Governance Indicators Predict Invasive Species Risk from Trade. *Proceedings. Biological sciences,* 283(1832).

Brown, E. S. & Swaine, G. 1965. Virus Disease of the African Armyworm, Spodoptera Exempta (Wlk.). *Bulletin of Entomological Research,* 56(1)**,** 95-116.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. & Wittwer, C. T. 2009. The Miqe Guidelines: Minimum Information for Publication of Quantitative Real-Time Pcr Experiments. *Clinical Chemistry,* 55(4)**,** 611.

Buès, R., Bouvier, J. C. & Boudinhon, L. 2005. Insecticide Resistance and Mechanisms of Resistance to Selected Strains of Helicoverpa Armigera (Lepidoptera: Noctuidae) in the South of France. *Crop Protection,* 24(9)**,** 814-820.

Bürgi, L. P. & Mills, N. J. 2014. Lack of Enemy Release for an Invasive Leafroller in California: Temporal Patterns and Influence of Host Plant Origin. *Biological Invasions,* 16(5)**,** 1021-1034.

Cabi. 2020. *Fall Armyworm (Faw) Portal* [Online]. Available: www.cabi.org/isc/fallarmyworm [Accessed 17/06/2020 2020].

Cabi. 2021. *Halyomorpha Halys (Brown Marmorated Stink Bug)* [Online]. Available: www.cabi.org/isc/datasheet/27377 [Accessed 20/05/2021.

Cabodevilla, O., Ibañez, I., Simón, O., Murillo, R., Caballero, P. & Williams, T. 2011. Occlusion Body Pathogenicity, Virulence and Productivity Traits Vary with Transmission Strategy in a Nucleopolyhedrovirus. *Biological Control,* 56(2)**,** 184-192.

Caniço, A., Mexia, A. & Santos, L. 2020. Seasonal Dynamics of the Alien Invasive Insect Pest Spodoptera Frugiperda Smith (Lepidoptera: Noctuidae) in Manica Province, Central Mozambique. *Insects,* 11(8).

Cankar, K., Štebih, D., Dreo, T., Žel, J. & Gruden, K. 2006. Critical Points of Dna Quantification by Real-Time Pcr – Effects of Dna Extraction Method and Sample Matrix on Quantification of Genetically Modified Organisms. *BMC Biotechnology,* 6(1)**,** 37.

Caprio, M. A. & Tabashnik, B. E. 1992. Gene Flow Accelerates Local Adaptation among Finite Populations: Simulating the Evolution of Insecticide Resistance. *Journal of Economic Entomology,* 85(3)**,** 611-620.

Castillejos, V., Trujillo, J., Ortega, L. D., Santizo, J. A., Cisneros, J., Penagos, D. I., Valle, J. & Williams, T. 2002. Granular Phagostimulant Nucleopolyhedrovirus Formulations for Control of *Spodoptera Frugiperda* in Maize. *Biological Control,* 24(3)**,** 300-310.

Cesari, M., Maistrello, L., Ganzerli, F., Dioli, P., Rebecchi, L. & Guidetti, R. 2015. A Pest Alien Invasion in Progress: Potential Pathways of Origin of the Brown Marmorated Stink Bug Halyomorpha Halys Populations in Italy. *Journal of Pest Science,* 88(1)**,** 1-7.

Chang, H., Guo, J. L., Fu, X. W., Hou, Y. M. & Wu, K. M. 2019. Orientation Behavior and Regulatory Gene Expression Profiles in Migratory Agrotis Ipsilon (Lepidoptera: Noctuidae). *Journal of insect behavior,* 32(1)**,** 59-67.

Chapman, J. W. 2012. Recent Insights from Entomological Radar Studies of High-Altitude Insect Migration. *Integr. Comp. Biol.,* 52**,** E28-E28.

Chapman, J. W., Reynolds, D. R., Hill, J. K., Sivell, D., Smith, A. D. & Woiwod, I. P. 2008. A Seasonal Switch in Compass Orientation in a High-Flying Migrant Moth. *Current Biology,* 18(19)**,** R908-R909.

Chapman, J. W., Reynolds, D. R. & Wilson, K. 2015. Long-Range Seasonal Migration in Insects: Mechanisms, Evolutionary Drivers and Ecological Consequences. *Ecology Letters,* 18(3)**,** 287-302.

Chen, Y.-H., Zhao, L., Pang, L.-R., Li, X.-Y., Weng, S.-P. & He, J.-G. 2012. Identification and Characterization of Inositol-Requiring Enzyme-1 and X-Box Binding Protein 1, Two Proteins Involved in the Unfolded Protein Response of Litopenaeus Vannamei. *Developmental and Comparative Immunology,* 38(1)**,** 66-77.

Cheng, T., Lin, P., Huang, L., Wu, Y., Jin, S., Liu, C. & Xia, Q. 2016a. Genome-Wide Analysis of Host Responses to Four Different Types of Microorganisms in Bombyx Mori (Lepidoptera: Bombycidae).(Research Article). *Journal of Insect Science,* 16(1).

Cheng, Y., Luo, L., Sappington, T. W., Jiang, X., Zhang, L. & Frolov, A. N. 2016b. Onset of Oviposition Triggers Abrupt Reduction in Migratory Flight Behavior and Flight Muscle in the Female Beet Webworm, Loxostege Sticticalis. *PLOS ONE,* 11(11)**,** e0166859.

Cheng, Z., Tsuda, M., Kishita, Y., Sato, Y. & Aigaki, T. 2013. Impaired Energy Metabolism in a Drosophila Model of Mitochondrial Aconitase Deficiency. *Biochemical and Biophysical Research Communications,* 433(1)**,** 145-150.

Cipriano, G.-G., César, M. E.-B. & Miguel, A. L. 2013. Infectivity of a Sinaloa Native Isolate of Multicapsid Nuclear Polyhedrosis Virus (Sfmnpv) against Fall Armyworm, Spodoptera Frugiperda (Lepidoptera: Noctuidae). *Southwestern Entomologist,* 38(4)**,** 597-604.

Cokola, M. C., Mugumaarhahama, Y., Noël, G., Bisimwa, E. B., Bugeme, D. M., Chuma, G. B., Ndeko, A. B. & Francis, F. 2020. Bioclimatic Zonation and Potential Distribution of Spodoptera Frugiperda (Lepidoptera: Noctuidae) in South Kivu Province, Dr Congo. *BMC Ecology,* 20(1)**,** 66.

Conesa, A. & Gtz, S. 2005. Blast2go: A Universal Tool for Annotation, Visualization and Analysis in Functional Genomics Research. *Bioinformatics,* 21(18)**,** 3674-3676.

Constantine, K., Kansiime, M., Idah, M., Nunda, W., Chacha, D., Rware, H., Makale, F., Mulema, J., Godwin, J., Williams, F., Edgington, S. & Day, R. 2020. Why Don't Smallholder Farmers in Kenya Use More Biopesticides? *Pest Management Science,* 76.

Cornell, H. V. & Hawkins, B. A. 1993. Accumulation of Native Parasitoid Species on Introduced Herbivores: A Comparison of Hosts as Natives and Hosts as Invaders. *The American Naturalist,* 141(6)**,** 847-865.

Cory, J. S. & Hoover, K. 2006. Plant-Mediated Effects in Insect–Pathogen Interactions. *Trends in Ecology & Evolution,* 21(5)**,** 278-286.

Cory, J. S. & Myers, J. H. 2003. The Ecology and Evolution of Insect Baculoviruses. *Annual Review of Ecology, Evolution, and Systematics,* 34(1)**,** 239-272.

Costa, J.-M., Alanio, A., Moukoury, S., Clairet, V., Debruyne, M., Poveda, J.-D. & Bretagne, S. 2013. Direct Genotyping of Toxoplasma Gondii from Amniotic Fluids Based on B1 Gene Polymorphism Using Minisequencing Analysis. *BMC Infectious Diseases,* 13(1)**,** 552.

Cotter, S., Reavey, C., Tummala, Y., Randall, J., Holdbrook, B., Ponton, F., Simpson, S., Smith, J. & Wilson, K. 2019. Diet Modulates the Relationship between Immune Gene Expression and Functional Immune Responses. *Insect Biochemistry and Molecular Biology,* 109.

Crall, A. W., Newman, G. J., Jarnevich, C. S., Stohlgren, T. J., Waller, D. M. & Graham, J. 2010. Improving and Integrating Data on Invasive Species Collected by Citizen Scientists. *Biological Invasions,* 12(10)**,** 3419-3428.

Crnokrak, P. & Roff, D. 1995. Fitness Differences Associated with Calling Behaviour in the Two Wing Morphs of Male Sand Crickets, *Gryllus Firmus*. *Anim. Behav.,* 50**,** 1475-1481.

Crnokrak, P. & Roff, D. A. 1998. The Genetic Basis of the Trade-Off between Calling and Wing Morph in Males of the Cricket *Gryllus Firmus*. *Evolution,* 52(4)**,** 1111-1118.

Crnokrak, P. & Roff, D. A. 2002. Trade-Offs to Flight Capability in *Gryllus Firmus* : The Influence of Whole-Organism Respiration Rate on Fitness. *Journal of Evolutionary Biology,* 15(3)**,** 388-398.

Crowder, D. W. & Snyder, W. E. 2010. Eating Their Way to the Top? Mechanisms Underlying the Success of Invasive Insect Generalist Predators. *Biological invasions,* 12(9)**,** 2857-2876.

Crémazy, F., Berta, P. & Girard, F. 2001. Genome-Wide Analysis of Sox Genes in Drosophila Melanogaster. *Mechanisms of Development,* 109(2)**,** 371-375.

Cugala, D., Schulthess, F., Ogol, C. P. O. & Omwega, C. O. 2006. Assessment of the Impact of Natural Enemies on Stemborer Infestations and Yield Loss in Maize Using Selected Insecticides in Mozambique. *Annales de la Société entomologique de France (N.S.),* 42(3-4)**,** 503-510.

Daehwan, K., Ben, L. & Steven, L. S. 2015. Hisat: A Fast Spliced Aligner with Low Memory Requirements. *Nature Methods,* 12(4).

Davidson, A., Jennions, M. & Nicotra, A. B. 2011. Do Invasive Species Show Higher Phenotypic Plasticity Than Native Species and, If So, Is It Adaptive? A Meta-Analysis. *Ecology Letters,* 14**,** 419-431.

Davis, A. K., Smith, F. M. & Ballew, A. M. 2020. A Poor Substitute for the Real Thing: Captive-Reared Monarch Butterflies Are Weaker, Paler and Have Less Elongated Wings Than Wild Migrants. *Biology Letters,* 16(4)**,** 20190922.

De Zoysa, M. D. & Yu, Y.-T. 2017. Posttranscriptional Rna Pseudouridylation. *The Enzymes,* 41**,** 151-167.

Derks, M., Smit, S., Salis, L., Schijlen, E., Bossers, A., Mateman, C., Pijl, A., Ridder, D., Groenen, M., Visser, M. & Megens, H.-J. 2015. *The Genome of Winter Moth (Operophtera Brumata) Provides a Genomic Perspective on Sexual Dimorphism and Phenology*.

Destoumieux-Garzón, D., Brehelin, M., Bulet, P., Boublik, Y., Girard, P.-A., Baghdiguian, S., Zumbihl, R. & Escoubas, J.-M. 2009. Spodoptera Frugiperda X-Tox Protein, an Immune Related Defensin Rosary, Has Lost the Function of Ancestral Defensins (Spod11tox Isn't Cleaved in Amp). *PLoS ONE,* 4(8)**,** e6795.

Dingle, H. 2014. *Migration : The Biology of Life on the Move,* Oxford, United Kingdom: Oxford University Press.

Dolezal, T., Krejčová, G., Bajgar, A., Nedbalova, P. & Strasser, P. 2019. Molecular Regulations of Metabolism During Immune Response in Insects. *Insect Biochemistry and Molecular Biology,* 109.

Dong, X., Li, Q. & Zhang, H. 2016. The Noa Gene Is Functionally Linked to the Activation of the Toll/Imd Signaling Pathways in Bactrocera Dorsalis (Hendel). *Developmental and Comparative Immunology,* 55**,** 233-240.

Dorhout, D. L., Sappington, T. W. & Rice, M. E. 2008. Evidence for Obligate Migratory Flight Behavior in Young European Corn Borer (Lepidoptera: Crambidae) Females. *Environmental Entomology,* 37(5)**,** 1280-1290.

Dringen, R. 2000. Metabolism and Functions of Glutathione in Brain. *Progress in Neurobiology,* 62(6)**,** 649-671.

Du Plessis, H., Schlemmer, M.-L. & Van Den Berg, J. 2020. The Effect of Temperature on the Development of Spodoptera Frugiperda (Lepidoptera: Noctuidae). *Insects,* 11(4)**,** 228.

Dumas, P., Legeai, F., Lemaitre, C., Scaon, E., Orsucci, M., Labadie, K., Gimenez, S., Clamens, A.-L., Henri, H., Vavre, F., Aury, J.-M., Fournier, P., Kergoat, G. & D'alençon, E. 2015. Spodoptera Frugiperda (Lepidoptera: Noctuidae) Host-Plant Variants: Two Host Strains or Two Distinct Species? *An International Journal of Genetics and Evolution,* 143(3)**,** 305-316.

Duneau, D. F., Kondolf, H. C., Im, J. H., Ortiz, G. A., Chow, C., Fox, M. A., Eugénio, A. T., Revah, J., Buchon, N. & Lazzaro, B. P. 2017. The Toll Pathway Underlies Host Sexual Dimorphism in Resistance to Both Gram-Negative and Gram-Positive Bacteria in Mated Drosophila. *BMC biology,* 15(1)**,** 124-124.

Dällenbach, L. J., Glauser, A., Lim, K. S., Chapman, J. W. & Menz, M. H. M. 2018. Higher Flight Activity in the Offspring of Migrants Compared to Residents in a Migratory Insect. *Proceedings. Biological sciences,* 285(1881)**,** 20172829.

Earl, D. A. & Vonholdt, B. M. 2012. Structure Harvester: A Website and Program for Visualizing Structure Output and Implementing the Evanno Method. *Conservation Genetics Resources,* 4(2)**,** 359-361.

Early, R., González-Moreno, P., Murphy, S. T. & Day, R. 2018. Forecasting the Global Extent of Invasion of the Cereal Pest Spodoptera Frugiperda, the Fall Armyworm. *NeoBiota,* 40**,** 25-50.

Ehler, L. E. 2006. Integrated Pest Management (Ipm): Definition, Historical Development and Implementation, and the Other Ipm. *Pest Management Science,* 62(9)**,** 787-789.

Ehrenfeld, J. G. 2010. Ecosystem Consequences of Biological Invasions. *Annu. Rev. Ecol. Evol. Syst.,* 41**,** 59-80.

Eikenaar, C., Hessler, S. & Hegemann, A. 2020. Migrating Birds Rapidly Increase Constitutive Immune Function During Stopover. *Royal Society Open Science,* 7(2)**,** 192031.

Elvitigala, D. A. S., Premachandra, H. K. A., Whang, I., Yeo, S.-Y., Choi, C. Y., Noh, J. K. & Lee, J. 2015. Molecular Cloning, Expression and Functional Characterization of a Teleostan Cytokine-Induced Apoptosis Inhibitor from Rock Bream (Oplegnathus Fasciatus). *Developmental and Comparative Immunology,* 52(1)**,** 48-57.

Eubanks, M. D. & Denno, R. F. 2000. Health Food Versus Fast Food: The Effects of Prey Quality and Mobility on Prey Selection by a Generalist Predator and Indirect Interactions among Prey Species. *Ecological Entomology,* 25(2)**,** 140-146.

European Environment Agency 2012. The Impacts of Invasive Alien Species in Europe. Luxemburg.

Fao. 2010. *Crop Calendar* [Online]. Food and Agriculture Organisation of the United Nations. Available: www.fao.org/agriculture/seed/cropcalendar/welcome.do [Accessed 16/02/2021.

Fao. 2021. *Famews Global Action for Fall Armyworm Control* [Online]. Available: http://www.fao.org/fall-armyworm/monitoring-tools/famews-global-platform/en/ [Accessed 20/05/2021.

Farias, J. R., Andow, D. A., Horikoshi, R. J., Sorgatto, R. J., Fresia, P., Dos Santos, A. C. & Omoto, C. 2014. Field-Evolved Resistance to Cry1f Maize by Spodoptera Frugiperda (Lepidoptera: Noctuidae) in Brazil. *Crop Protection,* 64**,** 150-158.

Fenton, A. & Brockhurst, M. A. 2008. The Role of Specialist Parasites in Structuring Host Communities. *Ecological Research,* 23(5)**,** 795-804.

Ferguson, H. J., Eaton, J. L. & Rogers, C. E. 1997. Larval Rearing Density Effects on Lipid Reserves and Wing-Loading in Fall Armyworm Adults (Lepidoptera: Noctuidae). *Journal of Agricultural Entomology,* 14(4)**,** 369-384.

Ferrandon, D., Imler, J.-L., Hetru, C. & A Hoffmann, J. 2007. *The Drosophila Systemic Immune Response: Sensing and Signalling During Bacterial and Fungal Infections*.

Ferreira, Á. G., Naylor, H., Esteves, S. S., Pais, I. S., Martins, N. E. & Teixeira, L. 2014. The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral Infection in Drosophila. *PLoS pathogens,* 10(12)**,** e1004507-e1004507.

Firake, D. M. & Behere, G. T. 2020. Natural Mortality of Invasive Fall Armyworm, Spodoptera Frugiperda (J. E. Smith) (Lepidoptera: Noctuidae) in Maize Agroecosystems of Northeast India. *Biological control,* 148**,** 104303.

Floate, K. D., Kyei-Poku, G. K. & Coghlin, P. C. 2006. Overview and Relevance of Wolbachia Bacteria in Biocontrol Research. *Biocontrol Science and Technology,* 16(8)**,** 767-788.

Flockhart, D. T. T., Wassenaar, L. I., Martin, T. G., Hobson, K. A., Wunder, M. B. & Norris, D. R. 2013. Tracking Multi-Generational Colonization of the Breeding Grounds by Monarch Butterflies in Eastern North America. *Proceedings: Biological Sciences,* 280(1768)**,** 1-8.

Food and Agriculture Organisation 2018. Integrated Management of the Fall Armyworm on Maize: A Guide for Farmer Field Schools in Africa. Rome, Italy: Food and Agriculture Organisation.

Food and Agriculture Organisation of the United Nations. 2021. *Global Action for Fall Armyworm Control* [Online]. Available: www.fao.org/fall-armyworm/monitoring-tools/faw-map/en/ [Accessed 01/10/2021.

Freedman, M. & Dingle, H. 2018. Wing Morphology in Migratory North American Monarchs: Characterizing Sources of Variation and Understanding Changes through Time. *Animal Migration,* 5**,** 61-73.

Fritzsche Mckay, A., Ezenwa, V. O. & Altizer, S. 2016. Unravelling the Costs of Flight for Immune Defenses in the Migratory Monarch Butterfly. *Integrative and Comparative Biology,* 56(2)**,** 278-289.

Fritzsche Mckay, A. & Hoye, B. J. 2016. Are Migratory Animals Superspreaders of Infection? *Integrative and Comparative Biology,* 56(2)**,** 260-267.

Fronza, E., Specht, A., Heinzen, H. & De Barros, N. M. 2017. Metarhizium (Nomuraea) Rileyi as Biological Control Agent. *Biocontrol Science and Technology,* 27(11)**,** 1243-1264.

Fu, C. & Whitfield, C. W. 2012. Genes Associated with Honey Bee Behavioral Maturation Affect Clock-Dependent and -Independent Aspects of Daily Rhythmic Activity in Fruit Flies. *PLOS ONE,* 7(5)**,** e29157.

Furukawa, S., Tanaka, K., Fukatsu, T. & Sasaki, T. 2008. In-Vitro Infection of *Wolbachia* in Insect Cell Lines. *Applied Entomology and Zoology,* 43(4)**,** 519-525.

Fuxa, J. R. 2004. Ecology of Insect Nucleopolyhedroviruses. *Agriculture, Ecosystems & Environment,* 103(1)**,** 27-43.

Fuxa, J. R. & Richter, A. R. 2001. Quantification of Soil-to-Plant Transport of Recombinant Nucleopolyhedrovirus: Effects of Soil Type and Moisture, Air Currents, and Precipitation. *Applied and environmental microbiology,* 67(11)**,** 5166-5170.

García-Banderas, D., Tamayo-Mejía, F., Pineda, S., Isaac Figueroa De La Rosa, J., Lasa, R., Manuel Chavarrieta-Yáñez, J., Gervasio-Rosas, E., Zamora-Avilés, N., Isabel Morales, S., Ramos-Ortiz, S., Valle, J. & Mabel Martínez-Castillo, A. 2020. Biological Characterization of Two Spodoptera Frugiperda Nucleopolyhedrovirus Isolates from Mexico and Evaluation of One Isolate in a Small-Scale Field Trial. *Biological Control***,** 104316.

Ge, S., Sun, X., He, W., Wyckhuys, K. A. G., He, L., Zhao, S., Zhang, H. & Wu, K. 2021. Potential Trade-Offs between Reproduction and Migratory Flight in Spodoptera Frugiperda. *Journal of Insect Physiology,* 132**,** 104248.

Geng, T., Lv, D.-D., Huang, Y.-X., Hou, C.-X., Qin, G.-X. & Guo, X.-J. 2016. Jak/Stat Signaling Pathway-Mediated Immune Response in Silkworm (Bombyx Mori) Challenged by Beauveria Bassiana. *Gene,* 595(1)**,** 69-76.

Gichuhi, J., Sevgan, S., Khamis, F., Van Den Berg, J., Du Plessis, H., Ekesi, S. & Herren, J. K. 2020. Diversity of Fall Armyworm, Spodoptera Frugiperda and Their Gut Bacterial Community in Kenya. *PeerJ,* 8**,** e8701.

Gitonga, Z. M., Chabi-Olaye, A., Mithöfer, D., Okello, J. J. & Ritho, C. N. 2010. Control of Invasive Liriomyza Leafminer Species and Compliance with Food Safety Standards by Small Scale Snow Pea Farmers in Kenya. *Crop Protection,* 29(12)**,** 1472-1477.

Go-Micro. 2021. *Fall Armyworm Detection* [Online]. Available: www.gomicro.co/faw/ [Accessed 20/05/2021.

Goergen, G., Kumar, P. L., Sankung, S. B., Togola, A. & Tamò, M. 2016. First Report of Outbreaks of the Fall Armyworm *Spodoptera Frugiperda* (J E Smith) (Lepidoptera, Noctuidae), a New Alien Invasive Pest in West and Central Africa. *PLoS ONE,* 11(10).

Goudet, J. 2005. Hierfstat, a Package for R to Compute and Test Hierarchical F-Statistics. *Molecular Ecology Notes,* 5(1)**,** 184-186.

Gouin, A., Bretaudeau, A., Nam, K., Gimenez, S., Aury, J.-M., Duvic, B., Hilliou, F., Durand, N., Montagné, N., Darboux, I., Kuwar, S., Chertemps, T., Siaussat, D., Bretschneider, A., Moné, Y., Ahn, S.-J., Hänniger, S., Grenet, A.-S., Neunemann, D., Maumus, F., Luyten, I., Labadie, K., Xu, W., Koutroumpa, F., Escoubas, J.-M., Llopis, A., Maïbèche-Coisne, M., Salasc, F., Tomar, A., Anderson, A., Khan, S., Dumas, P., Orsucci, M., Guy, J., Belser, C., Alberti, A., Noel, B., Couloux, A., Mercier, J., Nidelet, S., Dubois, E., Liu, N.-Y., Boulogne, I., Mirabeau, O., Le Goff, G., Gordon, K., Oakeshott, J., Consoli, F., Volkoff, A.-N., Fescemyer, H., Marden, J., Luthe, D., Herrero, S., Heckel, D., Wincker, P., Kergoat, G., Amselem, J., Quesneville, H., Groot, A., Jacquin-Joly, E., Nègre, N., Lemaitre, C., Legeai, F., D'alençon, E. & Fournier, P. 2017. Two Genomes of Highly Polyphagous Lepidopteran Pests (Spodoptera Frugiperda, Noctuidae) with Different Host-Plant Ranges. *Sci Rep,* 7(1)**,** 11816-11816.

Goulson, D. 2003. Can Host Susceptibility to Baculovirus Infection Be Predicted from Host Taxonomy or Life History? *Environmental Entomology,* 32(1)**,** 61-70.

Graham, R. & Wilson, K. 2012. Male-Killing Wolbachia and Mitochondrial Selective Sweep in a Migratory African Insect. *BMC Evolutionary Biology,* 12**,** 204.

Graham, R. I., Grzywacz, D., Mushobozi, W. L. & Wilson, K. 2012. Wolbachia in a Major African Crop Pest Increases Susceptibility to Viral Disease Rather Than Protects. *Ecology Letters,* 15(9)**,** 993-1000.

Grzywacz, D., Mushobozi, W., Parnell, M., Jolliffe, F. & Wilson, K. 2008. Evaluation of Spodoptera Exempta Nucleopolyhedrovirus (Spexnpv) for the Field Control of African Armyworm (Spodoptera Exempta) in Tanzania. *Crop Protection,* 27**,** 17-24.

Grzywacz, D., Stevenson, P. C., Mushobozi, W. L., Belmain, S. & Wilson, K. 2014. The Use of Indigenous Ecological Resources for Pest Control in Africa. *Food Security,* 6(1)**,** 71-86.

Guedes, R. N. C., Roditakis, E., Campos, M. R., Haddi, K., Bielza, P., Siqueira, H. A. A., Tsagkarakou, A., Vontas, J. & Nauen, R. 2019. Insecticide Resistance in the Tomato Pinworm Tuta Absoluta: Patterns, Spread, Mechanisms, Management and Outlook. *Journal of Pest Science,* 92(4)**,** 1329-1342.

Gunn, A. & Gatehouse, A. G. 1987. The Influence of Larval Phase on Metabolic Reserves, Fecundity and Lifespan of the African Armyworm Moth, *Spodoptera Exempta* (Walker) (Lepidoptera: Noctuidae). *Bulletin of Entomological Research,* 77(4)**,** 651-660.

Gunn, A., Gatehouse, A. G. & Woodrow, K. P. 1989. Trade-Off between Flight and Reproduction in the African Armyworm Moth, Spodoptera Exempta. *Physiological Entomology,* 14(4)**,** 419-427.

Guo, J.-W., Yang, F., Li, P., Liu, X.-D., Wu, Q.-L., Hu, G. & Zhai, B.-P. 2019. Female Bias in an Immigratory Population of Cnaphalocrocis Medinalis Moths Based on Field Surveys and Laboratory Tests. *Scientific Reports,* 9(1)**,** 18388.

Gupta, R. K., Gani, M., Kaul, V., Bhagat, R. M., Bali, K. & Samnotra, R. K. 2016. Field Evaluation of Lymantria Obfuscata Multiple Nucleopolyhedrovirus for the Management of Indian Gypsy Moth in Jammu & Kashmir, India. *Crop Protection,* 80**,** 149-158.

Gutiérrez-Moreno, R., Mota-Sanchez, D., Blanco, C. A., Whalon, M. E., Terán-Santofimio, H., Rodriguez-Maciel, J. C. & Difonzo, C. 2018. Field-Evolved Resistance of the Fall Armyworm (Lepidoptera: Noctuidae) to Synthetic Insecticides in Puerto Rico and Mexico. *Journal of Economic Entomology,* 112(2)**,** 792-802.

Gómez, J., Guevara, J., Cuartas, P., Espinel, C. & Villamizar, L. 2013. Microencapsulated Spodoptera Frugiperda Nucleopolyhedrovirus: Insecticidal Activity and Effect on Arthropod Populations in Maize. *Biocontrol Science and Technology,* 23(7)**,** 829-846.

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., Robles, M., Talón, M., Dopazo, J. & Conesa, A. 2008. High-Throughput Functional Annotation and Data Mining with the Blast2go Suite. *Nucleic Acids Research,* 36(10)**,** 3420-3435.

Hai-Xu, B., Hong-Fang, M., Xi-Xi, Z., Ming-Hui, P., Yu-Ping, L., Jun-Fang, S., Huan, W., Qun, L., Run-Xi, X., Yan-Qun, L. & Xing-Fu, J. 2017. Characterization of the Adult Head Transcriptome and Identification of Migration and Olfaction Genes in the Oriental Armyworm *Mythimna Separate*. *Scientific Reports,* 7(1).

Hajek, A. E. & Tobin, P. C. 2011. Introduced Pathogens Follow the Invasion Front of a Spreading Alien Host. *Journal of Animal Ecology,* 80(6)**,** 1217-1226.

Hall, R. J., Altizer, S. & Bartel, R. A. 2014. Greater Migratory Propensity in Hosts Lowers Pathogen Transmission and Impacts. *Journal of Animal Ecology,* 83(5)**,** 1068-1077.

Hall, T. A. 1999. Bioedit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/Nt. *Nucleic Acid Symposium Series,* 41**,** 95-98.

Hamilton, W. D. & Zuk, M. 1982. Heritable True Fitness and Bright Birds: A Role for Parasites? *Science,* 218(4570)**,** 384.

Hamma, T. & Ferré-D'amaré, A. R. 2010. The Box H/Aca Ribonucleoprotein Complex: Interplay of Rna and Protein Structures in Post-Transcriptional Rna Modification. *The Journal of Biological Chemistry,* 285(2)**,** 805-809.

Han, E. N. & Gatehouse, G. 1991. Effect of Temperature and Photoperiod on the Calling Behaviour of a Migratory Insect, the Oriental Armyworm Mythimna Separata. *Physiological Entomology,* 16(4)**,** 419-427.

Harvey, A. W. & Mallya, G. A. 1995. Predicting the Severity of Spodoptera Exempta (Lepidoptera: Noctuidae) Outbreak Seasons in Tanzania. *Bulletin of Entomological Research,* 85(4)**,** 479-487.

Harvey, K. J., Nipperess, D. A., Britton, D. R. & Hughes, L. 2012. Australian Family Ties: Does a Lack of Relatives Help Invasive Plants Escape Natural Enemies? *Biological Invasions,* 14(11)**,** 2423-2434.

Hassan, E.-S. M., Mesbah, I. I., Ali, F. A. & El-Shesheny, I. A. 2021. Prevalence, Population Dynamics and Associated Natural Enemies of Tomato Leafminer, Tuta Absoluta, in Egypt. *International Journal of Tropical Insect Science*.

He, L., Fu, X., Huang, Y., Shen, X., Sun, X. & Wu, K. 2018. Seasonal Patterns of Scotogramma Trifolii Rottemberg (Lepidoptera: Noctuidae) Migration across the Bohai Strait in Northern China. *Crop Protection,* 106**,** 34-41.

He, L., Ge, S., Zhang, H., He, W., Yan, R. & Wu, K. 2021. Photoregime Affects Development, Reproduction, and Flight Performance of the Invasive Fall Armyworm (Lepidoptera: Noctuidae) in China. *Environmental Entomology,* 50(2)**,** 367-381.

Hedrick, P. W. 2005. A Standardized Genetic Differentiation Measure. *Evolution,* 59(8)**,** 1633-1638.

Heimpel, G. E., Yang, Y., Hill, J. D. & Ragsdale, D. W. 2013. Environmental Consequences of Invasive Species: Greenhouse Gas Emissions of Insecticide Use and the Role of Biological Control in Reducing Emissions. *PLOS ONE,* 8(8)**,** e72293.

Herbert, R. I. & Mcgraw, E. A. 2018. The Nature of the Immune Response in Novel Wolbachia-Host Associations. *Symbiosis,* 74(3)**,** 225-236.

Hill, J. K. & Gatehouse, A. G. 2009. Effects of Temperature and Photoperiod on Development and Pre-Reproductive Period of the Silver Y Moth Autographa Gamma (Lepidoptera: Noctuidae). *Bulletin of Entomological Research,* 82(3)**,** 335-341.

Himmelstein, J., Ares, A., Gallagher, D. & Myers, J. 2017. A Meta-Analysis of Intercropping in Africa: Impacts on Crop Yield, Farmer Income, and Integrated Pest Management Effects. *International Journal of Agricultural Sustainability,* 15(1)**,** 1-10.

Hobson, K. A., Kusack, J. W. & Mora-Alvarez, B. X. 2021. Origins of Six Species of Butterflies Migrating through Northeastern Mexico: New Insights from Stable Isotope (Δ2h) Analyses and a Call for Documenting Butterfly Migrations. *Diversity,* 13(3)**,** 102.

Hoebeke, E. & Carter, M. E. 2003. Halyomorpha Halys (Stål) (Heteroptera: Pentatomidae): A Polyphagous Plant Pest from Asia Newly Detected in North America. *Proceedings of the Entomological Society of Washington,* 105**,** 225-237.

Holland, R. A., Wikelski, M. & Wilcove, D. S. 2006. How and Why Do Insects Migrate?(Perspective). *Science,* 313(5788)**,** 794-796.

Holt, R. D. & Lawton, J. H. 1994. The Ecological Consequences of Shared Natural Enemies. *Annual Review of Ecology and Systematics,* 25(1)**,** 495-520.

Huang, F., Qureshi, J. A., Meagher, R. L., Reisig, D. D., Head, G. P., Andow, D. A., Ni, X., Kerns, D., Buntin, G. D., Niu, Y., Yang, F. & Dangal, V. 2014. Cry1f Resistance in Fall Armyworm Spodoptera Frugiperda: Single Gene Versus Pyramided Bt Maize.(Research Article). *PLoS ONE,* 9(11).

Hudson, P. & Greenman, J. 1998. Competition Mediated by Parasites: Biological and Theoretical Progress. *Trends in Ecology & Evolution,* 13(10)**,** 387-390.

Huot, L., George, S., Girard, P.-A., Severac, D., Nègre, N. & Duvic, B. 2019. Spodoptera Frugiperda Transcriptional Response to Infestation by Steinernema Carpocapsae. *Scientific Reports,* 9(1)**,** 12879.

Ignoffo, C. M. 1992. Environmental Factors Affecting Persistence of Entomopathogens. *The Florida Entomologist,* 75(4)**,** 516-525.

Ignoffo, C. M. & Garcia, C. 1985. Host Spectrum and Relative Virulence of an Ecuadoran and a Mississippian Biotype of Nomuraea Rileyi. *Journal of Invertebrate Pathology,* 45(3)**,** 346-352.

Ignoffo, C. M., Hostetter, D. L., Sikorowski, P. P., Sutter, G. & Brooks, W. M. 1977. Inactivation of Representative Species of Entomopathogenic Viruses, a Bacterium, Fungus, and Protozoan by an Ultraviolet Light Source. *Environmental Entomology,* 6(3)**,** 411-415.

International Plant Protection Convention 2017. Occurence of Fall Armyworm (*Spodoptera Frugiperda*) in Malawi. Rome, Italy: International Plant Protection Convention.

Inyang, E. N., Butt, T. M., Ibrahim, L., Clark, S. J., Pye, B. J., Beckett, A. & Archer, S. 1998. The Effect of Plant Growth and Topography on the Acquisition of Conidia of the Insect Pathogen Metarhizium Anisopliae by Larvae of Phaedon Cochleariae. *Mycological Research,* 102(11)**,** 1365-1374.

Irving, P., Troxler, L., Heuer, T. S., Belvin, M., Kopczynski, C., Reichhart, J. M., Hoffmann, J. A. & Hetru, C. 2001. A Genome-Wide Analysis of Immune Responses in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America,* 98(26)**,** 15119-15124.

Isgren, E. & Andersson, E. 2020. An Environmental Justice Perspective on Smallholder Pesticide Use in Sub-Saharan Africa. *The Journal of Environment & Development,* 30(1)**,** 68-97.

Ishimaru, Y., Tomonari, S., Matsuoka, Y., Watanabe, T., Miyawaki, K., Bando, T., Tomioka, K., Ohuchi, H., Noji, S. & Mito, T. 2016. Tgf-B Signaling in Insects Regulates Metamorphosis Via Juvenile Hormone Biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America,* 113(20)**,** 5634-5639.

Jakobsson, M. & Rosenberg, N. A. 2007. Clumpp: A Cluster Matching and Permutation Program for Dealing with Label Switching and Multimodality in Analysis of Population Structure. *Bioinformatics,* 23(14)**,** 1801-1806.

Jakubowska, A. K., Vogel, H. & Herrero, S. 2013. Increase in Gut Microbiota after Immune Suppression in Baculovirus-Infected Larvae. *PLOS Pathogens,* 9(5)**,** e1003379.

Jeger, M., Bragard, C., Caffier, D., Candresse, T., Chatzivassiliou, E., Dehnen-Schmutz, K., Gilioli, G., Gregoire, J. C., Jaques Miret, J. A., Navarro, M. N., Niere, B., Parnell, S.,

Potting, R., Rafoss, T., Rossi, V., Urek, G., Van Bruggen, A., Van Der Werf, W., West, J., Winter, S., Gardi, C., Aukhojee, M. & Macleod, A. 2017. Pest Categorisation of *Spodoptera Frugiperda*. *EFSA Journal.*

Jepson, P. C., Guzy, M., Blaustein, K., Sow, M., Sarr, M., Mineau, P. & Kegley, S. 2014. Measuring Pesticide Ecological and Health Risks in West African Agriculture to Establish an Enabling Environment for Sustainable Intensification. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 369(1639)**,** 20130491.

Jiang, X., Luo, L., Zhang, L., Sappington, T. W. & Hu, Y. 2011. Regulation of Migration in Mythimna Separata (Walker) in China: A Review Integrating Environmental, Physiological, Hormonal, Genetic, and Molecular Factors. *Environmental Entomology,* 40(3)**,** 516-533.

Jiang, X. F., Luo, L. Z. & Sappington, T. W. 2010. Relationship of Flight and Reproduction in Beet Armyworm, Spodoptera Exigua (Lepidoptera: Noctuidae), a Migrant Lacking the Oogenesis-Flight Syndrome. *Journal of Insect Physiology,* 56(11)**,** 1631-1637.

Johns, S. & Shaw, A. 2016. Theoretical Insight into Three Disease-Related Benefits of Migration. *Population Ecology,* 58(1)**,** 213-221.

Johnson, C. G. 1969. *Migration and Dispersal of Insects by Flight,* London: London, Methuen.

Johnson, S. J. 1987. Migration and the Life History Strategy of the Fall Armyworm, Spodoptera Frugiperda in the Western Hemisphere. *International Journal of Tropical Insect Science,* 8(4-5-6)**,** 543-549.

Johnston, R. A., Paxton, K. L., Moore, F. R., Wayne, R. K. & Smith, T. B. 2016. Seasonal Gene Expression in a Migratory Songbird. *Molecular Ecology,* 25(22)**,** 5680-5691.

Jombart, T. 2008. Adegenet: A R Package for the Multivariate Analysis of Genetic Markers. *Bioinformatics,* 24(11)**,** 1403-1405.

Jones, C. M., Papanicolaou, A., Mironidis, G. K., Vontas, J., Yang, Y., Lim, K. S., Oakeshott, J. G., Bass, C. & Chapman, J. W. 2015. Genomewide Transcriptional Signatures of Migratory Flight Activity in a Globally Invasive Insect Pest. *Molecular Ecology,* 24(19)**,** 4901-4911.

Jones, C. M., Parry, H., Tay, W. T., Reynolds, D. R. & Chapman, J. W. 2019. Movement Ecology of Pest Helicoverpa: Implications for Ongoing Spread. *Annual Review of Entomology,* 64(1)**,** 277-295.

Jones, T. S., Godfray, H. C. J. & Van Veen, F. J. F. 2009. Resource Competition and Shared Natural Enemies in Experimental Insect Communities. *Oecologia,* 159(3)**,** 627-635.

Jost, L. O. U. 2008. Gst and Its Relatives Do Not Measure Differentiation. *Molecular Ecology,* 17(18)**,** 4015-4026.

Jupp, P. & Kemp, A. 1992. Aedes Albopictus and Other Mosquitoes Imported in Tires into Durban, South Africa. *Journal of the American Mosquito Control Association,* 8**,** 321-2.

Jyothi, P., Aralimarad, P., Wali, V., Dave, S., Bheemanna, M., Ashoka, J., Shivayogiyappa, P., Lim, K. S., Chapman, J. W. & Sane, S. P. 2021. Evidence for Facultative Migratory Flight Behavior in Helicoverpa Armigera (Noctuidae: Lepidoptera) in India. *PLOS ONE,* 16(1)**,** e0245665.

Kalilani, M. 2017. Declaration of State of Disaster in Districts Affected by Fall Armyworm Outbreak. Lilongwe, Malawi: Malawi government.

Kalule, T., Khan, Z. R., Bigirwa, G., Alupo, J., Okanya, S., Pickett, J. A. & Wadhams, L. J. 2006. Farmers' Perceptions of Importance, Control Practices and Alternative Hosts of Maize Stemborers in Uganda. *International Journal of Tropical Insect Science,* 26(2)**,** 71-77.

Kammer, A. E. & Heinrich, B. 1978. Insect Flight Metabolism. *In:* TREHERNE, J. E., BERRIDGE, M. J. & WIGGLESWORTH, V. B. (eds.) *Advances in Insect Physiology.* Academic Press.

Kamvar, Z. N., Tabima, J. F. & Grünwald, N. J. 2014. Poppr: An R Package for Genetic Analysis of Populations with Clonal, Partially Clonal, and/or Sexual Reproduction. *PeerJ,* 2**,** e281.

Kanehisa Laboratories 2021. Kegg: Kyoto Encylopedia of Genes and Genomes.

Kanost, M. R. & Jiang, H. 2015. Clip-Domain Serine Proteases as Immune Factors in Insect Hemolymph. *Current Opinion in Insect Science,* 11**,** 47-55.

Karthi, S. & Shivakumar, M. S. 2014. Circadian Clock Gene Is Involved in the Photoperiodic Response of the Spodoptera Litura Adults. *Biological Rhythm Research,* 45(5)**,** 731-737.

Kasambala Donga, T. & Meadow, R. 2018. Determination of Genetic Diversity in Chilo Partellus, Busseola Fusca, and Spodoptera Frugiperda Infesting Sugarcane in Southern Malawi Using Dna Barcodes. *Insects,* 9(3).

Keane, R. M. & Crawley, M. J. 2002. Exotic Plant Invasions and the Enemy Release Hypothesis. *Trends in Ecology & Evolution,* 17(4)**,** 164-170.

Keller, R. P., Geist, J., Jeschke, J. M. & Kühn, I. 2011. Invasive Species in Europe: Ecology, Status, and Policy. *Environmental Sciences Europe,* 23(1)**,** 23.

Kelly, C. D. 2018. The Causes and Evolutionary Consequences of Variation in Female Mate Choice in Insects: The Effects of Individual State, Genotypes and Environments. *Current Opinion in Insect Science,* 27**,** 1-8.

Kennedy, J. S. 1985. Migration: Behavioral and Ecological. *Contrib. Mar. Sci,* 27**,** 5-26.

Kim, D. & Shaw, A. K. 2021. Migration and Tolerance Shape Host Behaviour and Response to Parasite Infection. *Journal of Animal Ecology,* 90(10)**,** 2315-2324.

Kim, Y., Cha, S. J., Choi, H.-J. & Kim, K. 2017. Omega Class Glutathione S-Transferase: Antioxidant Enzyme in Pathogenesis of Neurodegenerative Diseases. *Oxidative medicine and cellular longevity,* 2017**,** 5049532-5049532.

Kim, Y. S., Ryu, J. H., Han, S. J., Choi, K. H., Nam, K. B., Jang, I. H., Lemaitre, B., Brey, P. T. & Lee, W. J. 2000. Gram-Negative Bacteria-Binding Protein, a Pattern Recognition Receptor for Lipopolysaccharide and Beta-1,3-Glucan That Mediates the Signaling for the Induction of Innate Immune Genes in Drosophila Melanogaster Cells. *The Journal of biological chemistry,* 275(42)**,** 32721-32727.

Kimberling, D. 2004. Lessons from History: Predicting Successes and Risks of Intentional Introductions for Arthropod Biological Control. *Biological Invasions,* 6(3)**,** 301-318.

King, E. G. 2011. The Evolutionary Genetics of Acquisition and Allocation in the Wing Dimorphic Cricket, *Gryllus Firmus*. *Evolution international journal of organic evolution.,* 65**,** 2273-2285.

Knop, E. & Reusser, N. 2012. Jack-of-All-Trades: Phenotypic Plasticity Facilitates the Invasion of an Alien Slug Species. *Proceedings. Biological sciences,* 279(1747)**,** 4668-1676.

Koala, L., Nikièma, A. S., Paré, A. B., Drabo, F., Toé, L. D., Belem, A. M. G., Boakye, D. A., Traoré, S. & Dabiré, R. K. 2019. Entomological Assessment of the Transmission Following Recrudescence of Onchocerciasis in the Comoé Valley, Burkina Faso. *Parasites & Vectors,* 12(1)**,** 34.

Koffi, D., Kyerematen, R., Eziah, V. Y., Agboka, K., Adom, M., Goergen, G. & Meagher, R. L. 2020. Natural Enemies of the Fall Armyworm, Spodoptera Frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) in Ghana. *The Florida entomologist,* 103(1)**,** 85.

Kondo, A. & Maeda, S. 1991. Host Range Expansion by Recombination of the Baculoviruses Bombyx Mori Nuclear Polyhedrosis Virus and Autographa Californica Nuclear Polyhedrosis Virus. *Journal of virology,* 65(7)**,** 3625-3632.

Koprivnikar, J. & Leung, T. L. F. 2015. Flying with Diverse Passengers: Greater Richness of Parasitic Nematodes in Migratory Birds. *Oikos,* 124(4)**,** 399-405.

Kouassi, L. N. G., Tsuda, K., Goto, C., Mukawa, S., Sakamaki, Y., Kusigemati, K. & Nakamura, M. 2009. Prevalence of Latent Virus in <I>Spodoptera Litura</I> (Fabricius)

(Lepidoptera: Noctuidae) and Its Activation by a Heterologous Virus. *Applied Entomology and Zoology,* 44(1)**,** 95-102.

Kriticos, D. J., Ota, N., Hutchison, W. D., Beddow, J., Walsh, T., Tay, W. T., Borchert, D. M., Paula-Moraes, S. V., Czepak, C. & Zalucki, M. P. 2015. The Potential Distribution of Invading Helicoverpa Armigera in North America: Is It Just a Matter of Time? *PloS one,* 10(3)**,** e0119618-e0119618.

Kvist, J., Mattila, A. L. K., Somervuo, P., Ahola, V., Koskinen, P., Paulin, L., Salmela, L., Fountain, T., Rastas, P., Ruokolainen, A., Taipale, M., Holm, L., Auvinen, P., Lehtonen, R., Frilander, M. J. & Hanski, I. 2015. Flight-Induced Changes in Gene Expression in the Glanville Fritillary Butterfly. *Molecular Ecology,* 24(19)**,** 4886-4900.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M. & Goettel, M. S. 2015a. Insect Pathogens as Biological Control Agents: Back to the Future. *J Invertebr Pathol,* 132**,** 1-41.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M. & Goettel, M. S. 2015b. Insect Pathogens as Biological Control Agents: Back to the Future. *Journal of Invertebrate Pathology,* 132**,** 1-41.

Lalitha, K., Karthi, S., Vengateswari, G., Karthikraja, R., Perumal, P. & Shivakumar, M. S. 2018. Effect of Entomopathogenic Nematode of Heterorhabditis Indica Infection on Immune and Antioxidant System in Lepidopteran Pest Spodoptera Litura (Lepidoptera: Noctuidae). *Journal of Parasitic Diseases,* 42(2)**,** 204-211.

Landwehr, A. 2021. Benefits of Baculovirus Use in Ipm Strategies for Open Field and Protected Vegetables. *Frontiers in Sustainable Food Systems,* 4.

Lang, M., Braun, C. L., Kanost, M. R. & Gorman, M. J. 2012. Multicopper Oxidase-1 Is a Ferroxidase Essential for Iron Homeostasis in Drosophila Melanogaster. *Proceedings of the National Academy of Sciences of the United States of America,* 109(33)**,** 13337-13342.

Lei, C., Yang, J., Wang, J., Hu, J. & Sun, X. 2020. Molecular and Biological Characterization of Spodoptera Frugiperda Multiple Nucleopolyhedrovirus Field Isolate and Genotypes from China. *Insects,* 11(11).

Lekei, E. E., Ngowi, A. V. & London, L. 2014. Farmers' Knowledge, Practices and Injuries Associated with Pesticide Exposure in Rural Farming Villages in Tanzania. *BMC Public Health,* 14(1)**,** 389.

Lesieur, V. & Farinha, A. O. 2021. Responses of Native Egg Parasitoids to the Invasive Seed Bug Leptoglossus Occidentalis. *Agricultural and Forest Entomology,* n/a(n/a).

Letellier, E. & Haan, S. 2016. Socs2: Physiological and Pathological Functions. *Frontiers in Bioscience - Elite,* 8(1)**,** 189-204.

Leung, B., Roura, N., Bacher, S., Heikkilä, J., Brotons, L., Burgman, M., Dehnen-Schmutz, K., Essl, F., Hulme, P., Richardson, D., Sol, D., Vilà, M. & Rejmanek, M. 2012. Teasing Apart Alien Species Risk Assessments: A Framework for Best Practices. *Ecology letters,* 15**,** 1475-93.

Lezama-Gutiérrez, R., Hamm, J. J., Molina-Ochoa, J., López-Edwards, M., Pescador-Rubio, A., González-Ramirez, M. & Styer, E. L. 2001. Occurrence of Entomopathogens of Spodoptera Frugiperda (Lepidoptera: Noctuidae) in the Mexican States of Michoacán, Colima, Jalisco and Tamaulipas. *The Florida Entomologist,* 84(1)**,** 23-30.

Li, H. & Durbin, R. 2009. Fast and Accurate Short Read Alignment with Burrows–Wheeler Transform. *Bioinformatics,* 25(14)**,** 1754-1760.

Liao, Y., Smyth, G. K. & Shi, W. 2014. Featurecounts: An Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features. *Bioinformatics,* 30(7)**,** 923-930.

Lichtenberg, E. & Olson, L. J. 2018. The Fruit and Vegetable Import Pathway for Potential Invasive Pest Arrivals. *PLOS ONE,* 13(2)**,** e0192280.

Lindstrom, R., Lindholm, P., Kallijarvi, J., Palgi, M., Saarma, M. & Heino, T. I. 2016. Exploring the Conserved Role of Manf in the Unfolded Protein Response in Drosophila

Melanogaster.(Mesencephalic Astrocyte-Derived Neurotrophic Factor)(Report). 11(3).
Lipper, C. H., Paddock, M. L., Onuchic, J. N., Mittler, R., Nechushtai, R. & Jennings, P. A. 2015. Cancer-Related Neet Proteins Transfer 2fe-2s Clusters to Anamorsin, a Protein Required for Cytosolic Iron-Sulfur Cluster Biogenesis. *PLOS ONE,* 10(10)**,** e0139699.
Liu, W., Liu, J., Lu, Y., Gong, Y., Zhu, M., Chen, F., Liang, Z., Zhu, L., Kuang, S., Hu, X., Cao, G., Xue, R. & Gong, C. 2015. Immune Signaling Pathways Activated in Response to Different Pathogenic Micro-Organisms in Bombyx Mori. *Molecular Immunology,* 65(2)**,** 391-397.
Lorenz, M. W. 2007. Oogenesis-Flight Syndrome in Crickets: Age-Dependent Egg Production, Flight Performance, and Biochemical Composition of the Flight Muscles in Adult Female *Gryllus Bimaculatus*. *Journal of Insect Physiology,* 53(8)**,** 819-832.
Lounibos, L. P. 2002. Invasions by Insect Vectors of Human Disease. *Annual Review of Entomology,* 47(1)**,** 233-266.
Lourenço, A. P., Florecki, M. M., Simões, Z. L. P. & Evans, J. D. 2018. Silencing of Apis Mellifera Dorsal Genes Reveals Their Role in Expression of the Antimicrobial Peptide Defensin-1. *Insect Molecular Biology,* 0(0).
Love, M. I., Huber, W. & Anders, S. 2014. Moderated Estimation of Fold Change and Dispersion for Rna-Seq Data with Deseq2. *Genome Biology,* 15(12)**,** 550.
Lu, Y., Yuan, M., Gao, X., Kang, T., Zhan, S., Wan, H. & Li, J. 2013. Identification and Validation of Reference Genes for Gene Expression Analysis Using Quantitative Pcr in Spodoptera Litura (Lepidoptera: Noctuidae). *PLOS ONE,* 8(7)**,** e68059.
Lugo Ramos, J. S., Delmore, K. E. & Liedvogel, M. 2017. Candidate Genes for Migration Do Not Distinguish Migratory and Non-Migratory Birds. *Journal of Comparative Physiology A,* 203(6)**,** 383-397.
Luo, I., Li, G., Gao, Y. & Hu, Y. 1995. The Influence of Larval Rearing Density on Flight Capacity and Fecundity of Adult Oriental Armyworm, *Mythimna Separata* (Walker). *ACTA ENTOMOLOGICA SINICA,* 38(1)**,** 38-45.
Maisonhaute, J.-É., Labrie, G. & Lucas, E. 2017. Direct and Indirect Effects of the Spatial Context on the Natural Biocontrol of an Invasive Crop Pest. *Biological Control,* 106**,** 64-76.
Maistrello, L., Dioli, P., Bariselli, M., Mazzoli, G. L. & Giacalone-Forini, I. 2016. Citizen Science and Early Detection of Invasive Species: Phenology of First Occurrences of Halyomorpha Halys in Southern Europe. *Biological Invasions,* 18(11)**,** 3109-3116.
Mallapur, C., Naik, A., Hagari, S. & Lingappa, S. 2018. *Potentiality of Nomuraea Rileyi (Farlow) Samson against the Fall Armyworm, Spodoptera Frugiperda (J E Smith) Infesting Maize*.
Manfredini, F., Arbetman, M. & Toth, A. L. 2019. A Potential Role for Phenotypic Plasticity in Invasions and Declines of Social Insects. *Frontiers in Ecology and Evolution,* 7(375).
Manley, R., Boots, M. & Wilfert, L. 2015. Review: Emerging Viral Disease Risk to Pollinating Insects: Ecological, Evolutionary and Anthropogenic Factors. *Journal of Applied Ecology,* 52(2)**,** 331-340.
Marco, H. G., Šimek, P. & Gäde, G. 2020. Unique Members of the Adipokinetic Hormone Family in Butterflies and Moths (Insecta, Lepidoptera). *Frontiers in Physiology,* 11(1542).
Marques, J. T. & Imler, J.-L. 2016. The Diversity of Insect Antiviral Immunity: Insights from Viruses. *Current Opinion in Microbiology,* 32**,** 71-76.
Masaki, S. & Seno, E. 1990. Effect of Selection on Wing Dimorphism in the Ground Cricket *Dianemobius Fascipes* (Walker). *Boletin de sanidad vegetal. Plagas,* 20**,** 381-393.
Matalin, A. V. 2003. Variations in Flight Ability with Sex and Age in Ground Beetles (Coleoptera, Carabidae) of South-Western Moldova. *Pedobiologia - International Journal of Soil Biology,* 47(4)**,** 311-319.

Mclean, M. J., Bishop, P. J. & Nakagawa, S. 2012. Male Quality, Signal Reliability and Female Choice: Assessing the Expectations of Inter-Sexual Selection. *Journal of Evolutionary Biology,* 25(8)**,** 1513-1520.

Mcneil, J., Cox-Foster, D., Slavicek, J. & Hoover, K. 2010. Contributions of Immune Responses to Developmental Resistance in Lymantria Dispar Challenged with Baculovirus. *Journal of Insect Physiology,* 56(9)**,** 1167-1177.

Men, Q.-L., Chen, M.-H., Zhang, Y.-L. & Feng, J.-N. 2013. Genetic Structure and Diversity of a Newly Invasive Species, the Codling Moth, Cydia Pomonella (L.) (Lepidoptera: Tortricidae) in China. *Biological Invasions,* 15(2)**,** 447-458.

Mende, K., Petoukhova, O., Koulitchkova, V., Schaub Günter, A., Lange, U., Kaufmann, R. & Nowak, G. 2001. Dipetalogastin, a Potent Thrombin Inhibitor from the Blood-Sucking Insectdipetalogaster Maximus. *European Journal of Biochemistry,* 266(2)**,** 583-590.

Menz, M. H. M., Reynolds, D. R., Gao, B., Hu, G., Chapman, J. W. & Wotton, K. R. 2019. Mechanisms and Consequences of Partial Migration in Insects. *Frontiers in Ecology and Evolution,* 7(403).

Midega, C. A. O., Nyang'au, I. M., Pittchar, J., Birkett, M. A., Pickett, J. A., Borges, M. & Khan, Z. R. 2012. Farmers' Perceptions of Cotton Pests and Their Management in Western Kenya. *Crop Protection,* 42**,** 193-201.

Midega, C. A. O., Pittchar, J. O., Pickett, J. A., Hailu, G. W. & Khan, Z. R. 2018. A Climate-Adapted Push-Pull System Effectively Controls Fall Armyworm, *Spodoptera Frugiperda* (J E Smith), in Maize in East Africa. *Crop Protection,* 105**,** 10-15.

Minter, M., Pearson, A., Lim, K. S., Wilson, K., Chapman, J. W. & Jones, C. M. 2018. The Tethered Flight Technique as a Tool for Studying Life-History Strategies Associated with Migration in Insects. *Ecological Entomology,* 43(4)**,** 397-411.

Mitchell, C. E., Blumenthal, D., Jarošík, V., Puckett, E. E. & Pyšek, P. 2010. Controls on Pathogen Species Richness in Plants' Introduced and Native Ranges: Roles of Residence Time, Range Size and Host Traits. *Ecology Letters,* 13(12)**,** 1525-1535.

Mitterboeck, T. F., Liu, S., Adamowicz, S. J., Fu, J., Zhang, R., Song, W., Meusemann, K. & Zhou, X. 2017. Positive and Relaxed Selection Associated with Flight Evolution and Loss in Insect Transcriptomes. *GigaScience,* 6(10).

Montezano, D., Specht, A., Sosa-Gómez, D., Roque-Specht, V., Sousa-Silva, J., Paula-Moraes, S., Peterson, J. & Hunt, T. 2018. Host Plants of Spodoptera Frugiperda (Lepidoptera: Noctuidae) in the Americas.

Moreno-García, M., Vargas, V., Ramírez-Bello, I., Hernández-Martínez, G. & Lanz-Mendoza, H. 2015. Bacterial Exposure at the Larval Stage Induced Sexual Immune Dimorphism and Priming in Adult Aedes Aegypti Mosquitoes. *PLOS ONE,* 10(7)**,** e0133240.

Mueller, J. C., Pulido, F. & Kempenaers, B. 2011. Identification of a Gene Associated with Avian Migratory Behaviour. *Proceedings of the Royal Society B: Biological Sciences,* 278(1719)**,** 2848-2856.

Muriithi, B., Gathogo, N. G., Diiro, G., Mohamed, S. & Ekesi, S. 2020. Potential Adoption of Integrated Pest Management Strategy for Suppression of Mango Fruit Flies in East Africa: An Ex Ante and Ex Post Analysis in Ethiopia and Kenya. *Agriculture,* 10**,** 278.

Murúa, G. & Virla, E. 2004. Population Parameters of *Spodoptera Frugiperda* (Smith) (Lep.: Noctuidae) Fed on Corn and Two Predominant Grasess in Tucuman (Argentina). *Acta zoológica mexicana,* 20**,** 199-210.

Møller, A. P. & Erritzøe, J. 1998. Host Immune Defence and Migration in Birds. *Evolutionary Ecology,* 12(8)**,** 945-953.

Nagelkerke, N. J. D. 1991. A Note on a General Definition of the Coefficient of Determination. *Biometrika,* 78(3)**,** 691-692.

Nagoshi, R., Goergen, G., Tounou, K., Agboka, K., Koffi, D. & Meagher, R. 2018a. Analysis of Strain Distribution, Migratory Potential, and Invasion History of Fall Armyworm Populations in Northern Sub-Saharan Africa. *Sci Rep,* 8(1)**,** 3710-3710.

Nagoshi, R. & Meagher, R. 2008. Review of Fall Armyworm (Lepidoptera: Noctuidae) Genetic Complexity and Migration. *The Florida Entomologist,* 91(4)**,** 546-554.
Nagoshi, R. N., Adamczyk, J. J., Meagher, R. L., Gore, J. & Jackson, R. 2007. Using Stable Isotope Analysis to Examine Fall Armyworm (Lepidoptera: Noctuidae) Host Strains in a Cotton Habitat. *Journal of economic entomology,* 100(5)**,** 1569.
Nagoshi, R. N., Dhanani, I., Asokan, R., Mahadevaswamy, H. M., Kalleshwaraswamy, C. M., Sharanabasappa & Meagher, R. L. 2019a. Genetic Characterization of Fall Armyworm Infesting South Africa and India Indicate Recent Introduction from a Common Source Population. *PLOS ONE,* 14(5)**,** e0217755.
Nagoshi, R. N., Fleischer, S., Meagher, R. L., Hay-Roe, M., Khan, A., Murúa, M. G., Silvie, P., Vergara, C. & Westbrook, J. 2017a. Fall Armyworm Migration across the Lesser Antilles and the Potential for Genetic Exchanges between North and South American Populations. *PloS one,* 12(2)**,** e0171743.
Nagoshi, R. N., Goergen, G., Plessis, H. D., Van Den Berg, J. & Meagher, R. 2019b. Genetic Comparisons of Fall Armyworm Populations from 11 Countries Spanning Sub-Saharan Africa Provide Insights into Strain Composition and Migratory Behaviors. *Scientific Reports,* 9(1)**,** 8311.
Nagoshi, R. N., Goergen, G., Tounou, K. A., Agboka, K., Koffi, D. & Meagher, R. L. 2018b. Analysis of Strain Distribution, Migratory Potential, and Invasion History of Fall Armyworm Populations in Northern Sub-Saharan Africa. *Scientific Reports,* 8(1)**,** 3710.
Nagoshi, R. N., Htain, N. N., Boughton, D., Zhang, L., Xiao, Y., Nagoshi, B. Y. & Mota-Sanchez, D. 2020. Southeastern Asia Fall Armyworms Are Closely Related to Populations in Africa and India, Consistent with Common Origin and Recent Migration. *Scientific Reports,* 10(1)**,** 1421.
Nagoshi, R. N., Koffi, D., Agboka, K., Tounou, K. A., Banerjee, R., Jurat-Fuentes, J. L. & Meagher, R. L. 2017b. Comparative Molecular Analyses of Invasive Fall Armyworm in Togo Reveal Strong Similarities to Populations from the Eastern United States and the Greater Antilles.(Research Article)(Report). *PLoS ONE,* 12(7)**,** e0181982.
Nagoshi, R. N. & Meagher, R. L. 2016. Using Intron Sequence Comparisons in the Triose-Phosphate Isomerase Gene to Study the Divergence of the Fall Armyworm Host Strains. *Insect Molecular Biology,* 25(3)**,** 324-337.
Nagoshi, R. N., Meagher, R. L. & Jenkins, D. A. 2010. Puerto Rico Fall Armyworm Has Only Limited Interactions with Those from Brazil or Texas but Could Have Substantial Exchanges with Florida Populations. *Journal of Economic Entomology,* 103(2)**,** 360-367.
Nagoshi, R. N., Rosas-García, N. M., Meagher, R. L., Fleischer, S. J., Westbrook, J. K., Sappington, T. W., Hay-Roe, M., Thomas, J. M. G. & Murúa, G. M. 2015. Haplotype Profile Comparisons between Spodoptera Frugiperda (Lepidoptera: Noctuidae) Populations from Mexico with Those from Puerto Rico, South America, and the United States and Their Implications to Migratory Behavior. *Journal of economic entomology,* 108(1)**,** 135.
Nakazawa, H., Tsuneishi, E., Ponnuvel, K. M., Furukawa, S., Asaoka, A., Tanaka, H., Ishibashi, J. & Yamakawa, M. 2004. Antiviral Activity of a Serine Protease from the Digestive Juice of Bombyx Mori Larvae against Nucleopolyhedrovirus. *Virology,* 321(1)**,** 154.
Nanda, S., Defalco, T. J., Hui Yong Loh, S., Phochanukul, N., Camara, N., Van Doren, M. & Russell, S. 2009. Sox100b, a Drosophila Group E Sox-Domain Gene, Is Required for Somatic Testis Differentiation. *Sexual Development,* 3(1)**,** 26-37.
Nations, U. 2020. The Sustainable Development Goals Report 2020. New York, USA.
Nayar, J. K. & Van Handel, E. 1971. Flight Performance and Metabolism of the Moth Spodoptera Frugiperda. *Journal of Insect Physiology,* 17(12)**,** 2475-2479.

Nayyar, N., Gracy, R. G., Ashika, T. R., Mohan, G., Swathi, R. S., Mohan, M., Chaudhary, M., Bakthavatsalam, N. & Venkatesan, T. 2021. Population Structure and Genetic Diversity of Invasive Fall Armyworm after 2 years of Introduction in India. *Scientific Reports,* 11(1)**,** 7760.

Nei, M. 1973. Analysis of Gene Diversity in Subdivided Populations. *Proceedings of the National Academy of Sciences,* 70(12)**,** 3321.

Neuenschwander, P. 2001. Biological Control of the Cassava Mealybug in Africa: A Review. *Biological Control,* 21(3)**,** 214-229.

Nicolas, E., Reichhart, J., Hoffmann, J. & Lemaitre, B. 1998. In Vivo Regulation of the I B Homologuecactus During the Immune Response Ofdrosophila. *Journal of Biological Chemistry,* 273**,** 10463-10469.

Niitepõld, K., Mattila, A. L. K., Harrison, P. J. & Hanski, I. 2011. Flight Metabolic Rate Has Contrasting Effects on Dispersal in the Two Sexes of the Glanville Fritillary Butterfly. *Oecologia,* 165(4)**,** 847-854.

Niz, J. M., Salvador, R., Ferrelli, M. L., De Cap, A. S., Romanowski, V. & Berretta, M. F. 2020. Genetic Variants in Argentinean Isolates of Spodoptera Frugiperda Multiple Nucleopolyhedrovirus. *Virus Genes,* 56(3)**,** 401-405.

Nunn, C. L., Lindenfors, P., Pursall, E. R. & Rolff, J. 2009. On Sexual Dimorphism in Immune Function. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences,* 364(1513)**,** 61-69.

Oberhauser, K. S. 1988. Male Monarch Butterfly Spermatophore Mass and Mating Strategies. *Animal Behaviour,* 36(5)**,** 1384-1388.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P., O'hara, R. B., Simpson, G., Solymos, P., Stevenes, M. H. H. & Wagner, H. 2012. Vegan: Community Ecology Package. R Package Version 2.0-2.

Östman, Ö., Ekbom, B. & Bengtsson, J. 2003. Yield Increase Attributable to Aphid Predation by Ground-Living Natural Enemies in Spring Barley in Sweden. *Ecological Economics,* 45**,** 149-158.

Otim, M. H., Adumo Aropet, S., Opio, M., Kanyesigye, D., Nakelet Opolot, H. & Tek Tay, W. 2021. Parasitoid Distribution and Parasitism of the Fall Armyworm Spodoptera Frugiperda (Lepidoptera: Noctuidae) in Different Maize Producing Regions of Uganda. *Insects,* 12(2)**,** 121.

Paini, D. R., Sheppard, A. W., Cook, D. C., De Barro, P. J., Worner, S. P. & Thomas, M. B. 2016. Global Threat to Agriculture from Invasive Species. *Proceedings of the National Academy of Sciences,* 113(27)**,** 7575.

Pantopoulos, K. & Hentze, M. W. 1995. Rapid Responses to Oxidative Stress Mediated by Iron Regulatory Protein. *The EMBO Journal,* 14(12)**,** 2917-2924.

Paradis, E. 2010. Pegas: An R Package for Population Genetics with an Integrated–Modular Approach. *Bioinformatics,* 26(3)**,** 419-420.

Park, T. 1948. Interspecies Competition in Populations of Trilobium Confusum Duval and Trilobium Castaneum Herbst. *Ecological Monographs,* 18(2)**,** 265-307.

Parry, H. R., Paull, C. A., Zalucki, M. P., Ives, A. R., Hulthen, A. & Schellhorn, N. A. 2017. Estimating the Landscape Distribution of Eggs by Helicoverpa Spp., with Implications for Bt Resistance Management. *Ecological Modelling,* 365**,** 129-140.

Parsa, S., Morse, S., Bonifacio, A., Chancellor, T. C. B., Condori, B., Crespo-Pérez, V., Hobbs, S. L. A., Kroschel, J., Ba, M. N., Rebaudo, F., Sherwood, S. G., Vanek, S. J., Faye, E., Herrera, M. A. & Dangles, O. 2014. Obstacles to Integrated Pest Management Adoption in Developing Countries. *Proceedings of the National Academy of Sciences,* 111(10)**,** 3889-3894.

Pashley, D. P. 1986. Host-Associated Genetic Differentiation in Fall Armyworm (Lepidoptera: Noctuidae): A Sibling Species Complex? *Annals of the Entomological Society of America,* 79(6)**,** 898-904.

Pasyukova, E. G., Roshina, N. V. & Mackay, T. F. C. 2004. Shuttle Craft : A Candidate Quantitative Trait Gene for Drosophila Lifespan. *Aging Cell,* 3(5)**,** 297-307.

Paula, D. P., Togni, P. H. B., Costa, V. A., Souza, L. M., Sousa, A. A. T. C., Tostes, G. M., Pires, C. S. S. & Andow, D. A. 2021. Scrutinizing the Enemy Release Hypothesis: Population Effects of Parasitoids on Harmonia Axyridis and Local Host Coccinellids in Brazil. *BioControl,* 66(1)**,** 71-82.

Pavinato, V. A. C. A. M. S. A. L. P. F. A. Z. M. I. A. O. C. 2013. Microsatellite Markers for Genetic Studies of the Fall Armyworm, Spodoptera Frugiperda. *Genetics and molecular research : GMR,* 12.

Pearson, A. 2016. *The Interaction between Migration and Disease in the Fall Armyworm (Spodoptera Frugiperda).* Doctor of Philosophy, Lancaster.

Peh, K. 2010. Invasive Species in Southeast Asia: The Knowledge So Far. *Biodiversity and Conservation,* 19**,** 1083-1099.

Perez, S. M., Taylor, O. R. & Jander, R. 1997. A Sun Compass in Monarch Butterflies. *Nature,* 387(6628)**,** 29-29.

Pimentel, D., Mcnair, S., Janecka, J., Wightman, J., Simmonds, C., O'connell, C., Wong, E., Russel, L., Zern, J., Aquino, T. & Tsomondo, T. 2001. Economic and Environmental Threats of Alien Plant, Animal, and Microbe Invasions. *Agriculture, Ecosystems & Environment,* 84(1)**,** 1-20.

Plongthongkum, N., Kullawong, N., Panyim, S. & Tirasophon, W. 2007. Ire1 Regulated Xbp1 Mrna Splicing Is Essential for the Unfolded Protein Response (Upr) in Drosophila Melanogaster. *Biochemical and Biophysical Research Communications,* 354(3)**,** 789-794.

Posselt, G., Schwarz, H., Duschl, A. & Horejs-Hoeck, J. 2011. Suppressor of Cytokine Signaling 2 Is a Feedback Inhibitor of Tlr-Induced Activation in Human Monocyte-Derived Dendritic Cells. *The Journal of Immunology,* 187(6)**,** 2875.

Pratt, C. F., Constantine, K. L. & Murphy, S. T. 2017. Economic Impacts of Invasive Alien Species on African Smallholder Livelihoods. *Global Food Security,* 14**,** 31-37.

Pretty, J. & Bharucha, Z. P. 2015. Integrated Pest Management for Sustainable Intensification of Agriculture in Asia and Africa. *Insects,* 6(1)**,** 152-182.

Pritchard, J. K., Stephens, M. & Donnelly, P. 2000. Inference of Population Structure Using Multilocus Genotype Data. *Genetics,* 155(2)**,** 945-959.

Provost, B., Jouan, V., Hilliou, F., Delobel, P., Bernardo, P., Ravallec, M., Cousserans, F., Wajnberg, E., Darboux, I., Fournier, P., Strand, M. R. & Volkoff, A.-N. 2011. Lepidopteran Transcriptome Analysis Following Infection by Phylogenetically Unrelated Polydnaviruses Highlights Differential and Common Responses. *Insect Biochemistry and Molecular Biology,* 41(8)**,** 582-591.

Purkiss, T. & Lach, L. 2019. Pathogen Spillover from Apis Mellifera to a Stingless Bee. *Proceedings of the Royal Society B: Biological Sciences,* 286(1908)**,** 20191071.

Pyke, B., Rice, M., Sabine, B. & Zalucki, M. 1987. The Push-Pull Strategy-Behavioural Control of Heliothis. *Australian Cotton Grower,* 9(1)**,** 7-9.

Qbase+ 2017. Qbase+ Manual. qbase+.

Qiagen 2018. Dneasy Blood and Tissue Handbook.

Qin, J., Liu, Y., Zhang, L., Cheng, Y., Sappington, T. W. & Jiang, X. 2018. Effects of Moth Age and Rearing Temperature on the Flight Performance of the Loreyi Leafworm, Mythimna Loreyi (Lepidoptera: Noctuidae), in Tethered and Free Flight. *Journal of Economic Entomology,* 111(3)**,** 1243-1248.

R Core Team 2020. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Ramsden, M. W., Menéndez, R., Leather, S. R. & Wäckers, F. 2015. Optimizing Field Margins for Biocontrol Services: The Relative Role of Aphid Abundance, Annual Floral Resources, and Overwinter Habitat in Enhancing Aphid Natural Enemies. *Agriculture, Ecosystems & Environment,* 199**,** 94-104.

Rankin, M., Hampton, E. & Summy, K. 1994. Investigations of the Oogenesis-Flight Syndrome in *Anthonomus Grandis* (Coleoptera: Curculionidae) Using Tethered Flight Tests. *Journal of Insect Behavior,* 7(6)**,** 795-810.

Raymond, L., Plantegenest, M. & Vialatte, A. 2013. Migration and Dispersal May Drive to High Genetic Variation and Significant Genetic Mixing: The Case of Two Agriculturally Important, Continental Hoverflies *(E. Pisyrphus Balteatus And .S Phaerophoria Scripta)*. *Molecular Ecology,* 22(21)**,** 5329-5339.

Raymond, M. & Rousset, F. 1995. Genepop (Version 1.2): Population Genetics Software for Exact Tests and Ecumenicism. *Journal of Heredity,* 86(3)**,** 248-249.

Redman, E. M., Wilson, K. & Cory, J. S. 2016. Trade-Offs and Mixed Infections in an Obligate-Killing Insect Pathogen. *J Anim Ecol,* 85(5)**,** 1200-1209.

Reeson, A. F., Wilson, K., Gunn, A., Hails, R. S. & Goulson, D. 1998. Baculovirus Resistance in the Noctuid Spodoptera Exempta Is Phenotypically Plastic and Responds to Population Density. *Proceedings of the Royal Society. B, Biological sciences,* 265(1407)**,** 1787-1791.

Reiter, P. & Sprenger, D. 1987. The Used Tire Trade: A Mechanism for the Worldwide Dispersal of Container Breeding Mosquitoes. *Journal of the American Mosquito Control Association,* 3(3)**,** 494 - 501.

Rejesus, R. M. & Jones, M. S. 2020. Perspective: Enhancing Economic Evaluations and Impacts of Integrated Pest Management Farmer Field Schools (Ipm-Ffs) in Low-Income Countries. *Pest Management Science,* 76(11)**,** 3527-3536.

Rhainds, M. & Kettela, E. 2013. Oviposition Threshold for Flight in an Inter-Reproductive Migrant Moth. *Journal of Insect Behavior,* 26(6)**,** 850-859.

Ribak, G., Barkan, S. & Soroker, V. 2017. The Aerodynamics of Flight in an Insect Flight-Mill. *PLOS ONE,* 12(11)**,** e0186441.

Roff, Derek a. & Fairbairn, Daphne j. 2007a. Laboratory Evolution of the Migratory Polymorphism in the Sand Cricket: Combining Physiology with Quantitative Genetics. *Physiological and Biochemical Zoology,* 80(4)**,** 358-369.

Roff, D. A. & Fairbairn, D. J. 2007b. The Evolution and Genetics of Migration in Insects. *BioScience,* 57(2)**,** 155-164.

Rolff, J. 2002. Bateman's Principle and Immunity. *Proceedings. Biological sciences,* 269(1493)**,** 867.

Rolff, J., Armitage, S. A. O. & Coltman, D. W. 2005. Genetic Constraints and Sexual Dimorphism in Immune Defense. *Evolution,* 59(8)**,** 1844-1850.

Rose, D. J. W., Dewhurst, C. F. & Page, W. W. 2000. *The African Armyworm Handbook: The Status, Biology, Ecology, Epidemiology and Management of Spodoptera Exempta (Lepidoptera: Noctuidae)*: Natural Resources Institute.

Rose, D. J. W., Page, W. W., Dewhurst, C. F., Riley, J. R., Reynolds, D. R., Pedgley, D. E. & Tucker, M. R. 1985. Downwind Migration of the African Army Worm Moth, Spodoptera Exempta, Studied by Mark-and-Capture and by Radar. *Ecological Entomology,* 10(3)**,** 299-313.

Rosenberg, N. A. 2004. Distruct: A Program for the Graphical Display of Population Structure. *Molecular Ecology Notes,* 4(1)**,** 137-138.

Roshina, N. V., Symonenko, A. V., Krementsova, A. V., Trostnikov, M. V. & Pasyukova, E. G. 2014. Embryonic Expression of Shuttle Craft, a Drosophila Gene Involved in Neuron Development, Is Associated with Adult Lifespan. *Aging,* 6(12)**,** 1076-1093.

Roux, M. M., Pain, A., Klimpel, K. R. & Dhar, A. K. 2002. The Lipopolysaccharide and B-1,3-Glucan Binding Protein Gene Is Upregulated in White Spot Virus-Infected Shrimp (Penaeus Stylirostris). *Journal of Virology,* 76(14)**,** 7140-7149.

Roy, H. E., Brown, P. M. J., Adriaens, T., Berkvens, N., Borges, I., Clusella-Trullas, S., Comont, R. F., De Clercq, P., Eschen, R., Estoup, A., Evans, E. W., Facon, B., Gardiner, M. M., Gil, A., Grez, A. A., Guillemaud, T., Haelewaters, D., Herz, A., Honek, A., Howe, A. G., Hui, C., Hutchison, W. D., Kenis, M., Koch, R. L., Kulfan, J., Lawson Handley, L., Lombaert, E., Loomans, A., Losey, J., Lukashuk, A. O., Maes, D., Magro, A., Murray, K. M., Martin, G. S., Martinkova, Z., Minnaar, I. A., Nedved, O., Orlova-Bienkowskaja, M. J., Osawa, N., Rabitsch, W., Ravn, H. P., Rondoni, G., Rorke, S. L., Ryndevich, S. K., Saethre, M.-G., Sloggett, J. J., Soares, A. O., Stals, R., Tinsley, M. C., Vandereycken, A., Van Wielink, P., Viglášová, S., Zach, P., Zakharov, I. A., Zaviezo, T. & Zhao, Z. 2016. The Harlequin Ladybird, Harmonia Axyridis: Global Perspectives on Invasion History and Ecology. *Biological Invasions,* 18(4)**,** 997-1044.

Roy, H. E., Lawson Handley, L. J., Schönrogge, K., Poland, R. L. & Purse, B. V. 2011. Can the Enemy Release Hypothesis Explain the Success of Invasive Alien Predators and Parasitoids? *BioControl,* 56(4)**,** 451-468.

Roy, H. E. & Pell, J. K. 2000. Interactions between Entomopathogenic Fungi and Other Natural Enemies: Implications for Biological Control. *Biocontrol Science and Technology,* 10(6)**,** 737-752.

Rwanda, N. I. O. S. 2017. 2017 Seasonal Agricultural Survey. Kigali, Rwanda.

Sadekuzzaman, M., Gautam, N. & Kim, Y. 2017. A Novel Calcium-Independent Phospholipase A2 and Its Physiological Roles in Development and Immunity of a Lepidopteran Insect, Spodoptera Exigua. *Developmental and Comparative Immunology,* 77(C)**,** 210-220.

Saino, N., Bazzi, G., Gatti, E., Caprioli, M., Cecere, J. G., Possenti, C. D., Galimberti, A., Orioli, V., Bani, L., Rubolini, D., Gianfranceschi, L. & Spina, F. 2015. Polymorphism at the Clock Gene Predicts Phenology of Long-Distance Migration in Birds. *Molecular Ecology,* 24(8)**,** 1758-1773.

Sakai, A. K., Allendorf, F. W., Holt, J. S., Lodge, D. M., Molofsky, J., With, K. A., Baughman, S., Cabin, R. J., Cohen, J. E., Ellstrand, N. C., Mccauley, D. E., O'neil, P., Parker, I. M., Thompson, J. N. & Weller, S. G. 2001. The Population Biology of Invasive Species. *Annual Review of Ecology and Systematics,* 32(1)**,** 305-332.

Sappington, T. W., Fescemyer, H. W. & Showers, W. B. 1995. Lipid and Carbohydrate Utilization During Flight of the Migratory Moth, Agrotis Ipsilon (Lepidoptera: Noctuidae). *Archives of Insect Biochemistry and Physiology,* 29(4)**,** 397-414.

Sarr, O. M., Bal, A. B., Fossati-Gaschignard, O. & Gauthier, N. 2021. Effectiveness of Two Biopesticides against the Invasive Tomato Pest Tuta Absoluta. *Entomologia Experimentalis et Applicata,* n/a(n/a).

Sartori, M., Keller, L., Thomas, A. G. B. & Passera, L. 1992. Flight Energetics in Relation to Sexual Differences in the Mating Behaviour of a Mayfly, Siphlonurus Aestivalis. *Oecologia,* 92(2)**,** 172-176.

Schroeder, H., Majewska, A. & Altizer, S. 2020. Monarch Butterflies Reared under Autumn-Like Conditions Have More Efficient Flight and Lower Post-Flight Metabolism. *Ecological Entomology,* 45(3)**,** 562-572.

Schwarz, H., Posselt, G., Wurm, P., Ulbing, M., Duschl, A. & Horejs-Hoeck, J. 2013. Tlr8 and Nod Signaling Synergistically Induce the Production of Il-1β and Il-23 in Monocyte-Derived Dcs and Enhance the Expression of the Feedback Inhibitor Socs2. *Immunobiology,* 218(4)**,** 533-542.

Schwenke, R. A., Lazzaro, B. P. & Wolfner, M. F. 2016. Reproduction-Immunity Trade-Offs in Insects. *Annual review of entomology,* 61**,** 239-256.

Sereno, D., Müller, W. E. G., Bausen, M., Elkhooly, T. A., Markl, J. S. & Wiens, M. 2017. An Evolutionary Perspective on the Role of Mesencephalic Astrocyte-Derived Neurotrophic Factor (Manf): At the Crossroads of Poriferan Innate Immune and Apoptotic Pathways. *Biochemistry and Biophysics Reports,* 11**,** 161-173.

Seufi, A. M., Hafez, E. E. & Galal, F. H. 2011. Identification, Phylogenetic Analysis and Expression Profile of an Anionic Insect Defensin Gene, with Antibacterial Activity, from Bacterial-Challenged Cotton Leafworm, Spodoptera Littoralis.(Research Article)(Report). *BMC Molecular Biology,* 12**,** 47.

Shackelford, G., Steward, P. R., Benton, T. G., Kunin, W. E., Potts, S. G., Biesmeijer, J. C. & Sait, S. M. 2013. Comparison of Pollinators and Natural Enemies: A Meta-Analysis of Landscape and Local Effects on Abundance and Richness in Crops. *Biological Reviews,* 88(4)**,** 1002-1021.

Shapiro, D. I., Fuxa, J. R., Braymer, H. D. & Pashley, D. P. 1991. Dna Restriction Polymorphism in Wild Isolates of Spodoptera Frugiperda Nuclear Polyhedrosis Virus. *Journal of Invertebrate Pathology,* 58(1)**,** 96-105.

Sharanabasappa, Kalleshwaraswamy, C. M., Poorani, J., Maruthi, M. S., Pavithra, H. B. & Diraviam, J. 2019. Natural Enemies of <I>Spodoptera Frugiperda</I> (J. E. Smith) (Lepidoptera: Noctuidae), a Recent Invasive Pest on Maize in South India. *Florida Entomologist,* 102(3)**,** 619-623.

Shaw, A. K. & Binning, S. A. 2016. Migratory Recovery from Infection as a Selective Pressure for the Evolution of Migration. *The American Naturalist,* 187(4)**,** 491-501.

Shaw, A. K. & Binning, S. A. 2020. Recovery from Infection Is More Likely to Favor the Evolution of Migration Than Social Escape from Infection. *The Journal of animal ecology*.

Shibayama, H., Takai, E., Matsumura, I., Kouno, M., Morii, E., Kitamura, Y., Takeda, J. & Kanakura, Y. 2004. Identification of a Cytokine-Induced Antiapoptotic Molecule Anamorsin Essential for Definitive Hematopoiesis. *The Journal of Experimental Medicine,* 199(4)**,** 581-592.

Shikano, I., Ericsson, J. D., Cory, J. S. & Myers, J. H. 2010. Indirect Plant-Mediated Effects on Insect Immunity and Disease Resistance in a Tritrophic System. *Basic and Applied Ecology,* 11(1)**,** 15-22.

Shikano, I., Mccarthy, E. M., Elderd, B. D. & Hoover, K. 2017a. Plant Genotype and Induced Defenses Affect the Productivity of an Insect-Killing Obligate Viral Pathogen. *J Invertebr Pathol,* 148**,** 34-42.

Shikano, I., Shumaker, K. L., Peiffer, M., Felton, G. W. & Hoover, K. 2017b. Plant-Mediated Effects on an Insect–Pathogen Interaction Vary with Intraspecific Genetic Variation in Plant Defences. *Oecologia,* 183(4)**,** 1121-1134.

Shirata, N., Ikeda, M., Kamiya, K., Kawamura, S. & Kobayashi, M. 2004. Restriction of *Bombyx Mori* Nucleopolyhedrovirus (Npv) Replication by *Hyphantria Cunea* Npv in a Cell Line from *B. Mori*. *Journal of Insect Biotechnology and Sericology,* 73(1)**,** 23-33.

Sileshi, G. W., Gebeyehu, S. & Mafongoya, P. L. 2019. The Threat of Alien Invasive Insect and Mite Species to Food Security in Africa and the Need for a Continent-Wide Response. *Food Security,* 11(4)**,** 763-775.

Silva-Brandão, K. L., Horikoshi, R. J., Bernardi, D., Omoto, C., Figueira, A. & Brandão, M. M. 2017. Transcript Expression Plasticity as a Response to Alternative Larval Host Plants in the Speciation Process of Corn and Rice Strains of Spodoptera Frugiperda. *BMC genomics,* 18(1)**,** 792-792.

Sim, S. & Dimopoulos, G. 2010. Dengue Virus Inhibits Immune Responses in Aedes Aegypti Cells. *PloS one,* 5(5)**,** e10678-e10678.

Simmons, A. M. & Rogers, C. E. 1991. Dispersal and Seasonal Occurrence of Noctuidonema Guyanense, an Ectoparasitic Nematode of Adult Fall Armyworm (Lepidoptera: Noctuidae), in the United States. *Journal of Entomological Science,* 26(1)**,** 136-148.

Simon, O., Williams, T., Lopez-Ferber, M. & Caballero, P. 2004. Virus Entry or the Primary Infection Cycle Are Not the Principal Determinants of Host Specificity of Spodoptera Spp. Nucleopolyhedroviruses. *J Gen Virol,* 85(Pt 10)**,** 2845-55.

Simón, O., Williams, T., López-Ferber, M., Taulemesse, J.-M. & Caballero, P. 2008. Population Genetic Structure Determines Speed of Kill and Occlusion Body Production in Spodoptera Frugiperda Multiple Nucleopolyhedrovirus. *Biological Control,* 44(3)**,** 321-330.

Sisay, B., Simiyu, J., Malusi, P., Likhayo, P., Mendesil, E., Elibariki, N., Wakgari, M., Ayalew, G. & Tefera, T. 2018. First Report of the Fall Armyworm, Spodoptera Frugiperda (Lepidoptera: Noctuidae), Natural Enemies from Africa. *Journal of Applied Entomology,* 142(8)**,** 800-804.

Sisay, B., Simiyu, J., Mendesil, E., Likhayo, P., Ayalew, G., Mohamed, S., Subramanian, S. & Tefera, T. 2019. Fall Armyworm, Spodoptera Frugiperda Infestations in East Africa: Assessment of Damage and Parasitism. *Insects,* 10(7)**,** 195.

Snyder, W. E. & Evans, E. W. 2006. Ecological Effects of Invasive Arthropod Generalist Predators. *Annual Review of Ecology, Evolution, and Systematics,* 37(1)**,** 95-122.

Sokame, B. M., Rebaudo, F., Malusi, P., Subramanian, S., Kilalo, D. C., Juma, G. & Calatayud, P.-A. 2020a. Influence of Temperature on the Interaction for Resource Utilization between Fall Armyworm, Spodoptera Frugiperda (Lepidoptera: Noctuidae), and a Community of Lepidopteran Maize Stemborers Larvae. *Insects,* 11(2).

Sokame, B. M., Subramanian, S., Kilalo, D. C., Juma, G. & Calatayud, P.-A. 2020b. Larval Dispersal of the Invasive Fall Armyworm, Spodoptera Frugiperda, the Exotic Stemborer Chilo Partellus, and Indigenous Maize Stemborers in Africa. *Entomologia Experimentalis et Applicata,* 168(4)**,** 322-331.

Somerville, A. G. T., Gleave, K., Jones, C. M. & Reimer, L. J. 2019. The Consequences of Brugia Malayi Infection on the Flight and Energy Resources of Aedes Aegypti Mosquitoes. *Scientific Reports,* 9(1)**,** 18449.

Souza, M. L., Sanches, M. M., Souza, D. A. D., Faria, M., Espinel-Correal, C., Sihler, W. & Lopes, R. B. 2019. Within-Host Interactions of Metarhizium Rileyi Strains and Nucleopolyhedroviruses in Spodoptera Frugiperda and Anticarsia Gemmatalis (Lepidoptera: Noctuidae). *J Invertebr Pathol,* 162**,** 10-18.

Srinivasan, R., Malini, P. & Othim, S. T. O. 2018. Fall Armyworm in Africa: Which 'Race' Is in the Race, and Why Does It Matter? *Current Science,* 114(1)**,** 27-28.

Srinivasan, R., Sevgan, S., Ekesi, S. & Tamò, M. 2019. Biopesticide Based Sustainable Pest Management for Safer Production of Vegetable Legumes and Brassicas in Asia and Africa. *Pest Management Science,* 75(9)**,** 2446-2454.

Srygley, R. B. & Dudley, R. 2008. Optimal Strategies for Insects Migrating in the Flight Boundary Layer: Mechanisms and Consequences. *Integrative and Comparative Biology,* 48(1)**,** 119-133.

Srygley, R. B. & Lorch, P. D. 2011. Weakness in the Band: Nutrient-Mediated Trade-Offs between Migration and Immunity of Mormon Crickets, *Anabrus Simplex*. *Animal Behaviour,* 81(2)**,** 395-400.

Srygley, R. B., Lorch, P. D., Simpson, S. J. & Sword, G. A. 2009. Immediate Protein Dietary Effects on Movement and the Generalised Immunocompetence of Migrating Mormon Crickets *Anabrus Simplex* (Orthoptera: Tettigoniidae). *Ecological Entomology,* 34(5)**,** 663-668.

Stevens, L., Monroy, M. C., Rodas, A. G., Hicks, R. M., Lucero, D. E., Lyons, L. A. & Dorn, P. L. 2015. Migration and Gene Flow among Domestic Populations of the Chagas Insect Vector Triatoma Dimidiata (Hemiptera: Reduviidae) Detected by Microsatellite Loci. *Journal of Medical Entomology,* 52(3)**,** 419-428.

Stielow, J. B., Lévesque, C. A., Seifert, K. A., Meyer, W., Iriny, L., Smits, D., Renfurm, R., Verkley, G. J. M., Groenewald, M., Chaduli, D., Lomascolo, A., Welti, S., Lesage-

Meessen, L., Favel, A., Al-Hatmi, A. M. S., Damm, U., Yilmaz, N., Houbraken, J., Lombard, L., Quaedvlieg, W., Binder, M., Vaas, L. A. I., Vu, D., Yurkov, A., Begerow, D., Roehl, O., Guerreiro, M., Fonseca, A., Samerpitak, K., Van Diepeningen, A. D., Dolatabadi, S., Moreno, L. F., Casaregola, S., Mallet, S., Jacques, N., Roscini, L., Egidi, E., Bizet, C., Garcia-Hermoso, D., Martín, M. P., Deng, S., Groenewald, J. Z., Boekhout, T., De Beer, Z. W., Barnes, I., Duong, T. A., Wingfield, M. J., De Hoog, G. S., Crous, P. W., Lewis, C. T., Hambleton, S., Moussa, T. A. A., Al-Zahrani, H. S., Almaghrabi, O. A., Louis-Seize, G., Assabgui, R., Mccormick, W., Omer, G., Dukik, K., Cardinali, G., Eberhardt, U., De Vries, M. & Robert, V. 2015. One Fungus, Which Genes? Development and Assessment of Universal Primers for Potential Secondary Fungal Dna Barcodes. *Persoonia,* 35**,** 242-263.

Storer, N. P., Babcock, J. M., Schlenz, M., Meade, T., Thompson, G. D., Bing, J. W. & Huckaba, R. M. 2010. Discovery and Characterization of Field Resistance to Bt Maize: Spodoptera Frugiperda (Lepidoptera: Noctuidae) in Puerto Rico. *Journal of Economic Entomology,* 103(4)**,** 1031-1038.

Stroumbakis, N. D., Li, Z. & Tolias, P. P. 1996. A Homolog of Human Transcription Factor Nf-X1 Encoded by the Drosophila Shuttle Craft Gene Is Required in the Embryonic Central Nervous System. *Molecular and Cellular Biology,* 16(1)**,** 192.

Suarez, R. K. 2000. Energy Metabolism During Insect Flight: Biochemical Design and Physiological Performance. *Physiological and Biochemical Zoology,* 73(6)**,** 765-771.

Sun, J.-J., Lan, J.-F., Xu, J.-D., Niu, G.-J. & Wang, J.-X. 2016. Suppressor of Cytokine Signaling 2 (Socs2) Negatively Regulates the Expression of Antimicrobial Peptides by Affecting the Stat Transcriptional Activity in Shrimp Marsupenaeus Japonicus. *Fish and Shellfish Immunology,* 56**,** 473-482.

Sun, Y., Wang, Y., Liu, W., Zhou, J., Zeng, J., Wang, X., Jiang, Y., Li, D. & Qin, L. 2017. Upregulation of a Trypsin-Like Serine Protease Gene in Antheraea Pernyi (Lepidoptera: Saturniidae) Strains Exposed to Different Pathogens. *J. Econ. Entomol.,* 110(3)**,** 941-948.

Syed, Z. A., Härd, T., Uv, A. & Van Dijk-Härd, I. F. 2008. A Potential Role for Drosophila Mucins in Development and Physiology. *PLOS ONE,* 3(8)**,** e3041.

Syromyatnikov, M. Y., Chugreev, M. Y., Lopatin, A. V., Starkov, A. A. & Popov, V. N. 2015. Production of Reactive Oxygen Species by Flight Muscle Mitochondria of the Bumblebee (Bombus Terrestris L.). *Doklady Biochemistry and Biophysics,* 463(1)**,** 229-231.

Tadross, M., Suarez, P., Lotsch, A., Hachigonta, S., Mdoka, M., Unganai, L., Lucio, F., Kamdonyo, D. & Muchinda, M. 2009. Growing-Season Rainfall and Scenarios of Future Change in Southeast Africa: Implications for Cultivating Maize. *Climate Research - CLIMATE RES,* 40**,** 147-161.

Tadross, M. A., Hewitson, B. C. & Usman, M. T. 2005. The Interannual Variability of the Onset of the Maize Growing Season over South Africa and Zimbabwe. *Journal of Climate,* 18(16)**,** 3356-3372.

Takaoka, H. 2015. 5) Review of the Biology and Ecology of Adult Blackflies in Relation to the Transmission of Onchocerciasis in Guatemala. *Tropical medicine and health,* 43(Suppl)**,** 71-85.

Taniai, K. & Imanishi, S. 2004. A Clip-Domain Serine Protease Activating Cecropin B Promoter in a Silkworm Cell Line Capable of Pathogen-Associated Pattern recognition NCBI GenBank.

Tay, W. T., Soria, M. F., Walsh, T., Thomazoni, D., Silvie, P., Behere, G. T., Anderson, C. & Downes, S. 2013. A Brave New World for an Old World Pest: Helicoverpa Armigera (Lepidoptera: Noctuidae) in Brazil. *PLOS ONE,* 8(11)**,** e80134.

Taylor, R. A. J. 2010. Flight Performance of *Agrilus Planipennis* (Coleoptera: Buprestidae) on a Flight Mill and in Free Flight. *Journal of insect behavior,* 23**,** 128-148.

Teo, L.-H., Fescemyer, H. W., Woodring, J. P. & Hammond, A. M. 1987. Carbohydrate and Fatty Acid Titres During Flight of the Migrant Noctuid Moth, Anticarsia Gemmatalis Hübner. *Insect Biochemistry,* 17(6)**,** 777-781.

Thiem, S. M. & Cheng, X.-W. 2009. Baculovirus Host-Range. *Virologica Sinica,* 24(5)**,** 436.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994. Clustal W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. *Nucleic acids research,* 22(22)**,** 4673-4680.

Tinnert, J., Hellgren, O., Lindberg, J., Koch-Schmidt, P. & Forsman, A. 2016. Population Genetic Structure, Differentiation, and Diversity in Tetrix Subulata Pygmy Grasshoppers: Roles of Population Size and Immigration. *Ecology and evolution,* 6(21)**,** 7831-7846.

Torchin, M. E., Lafferty, K. D., Dobson, A. P., Mckenzie, V. J. & Kuris, A. M. 2003. Introduced Species and Their Missing Parasites. *Nature,* 421(6923)**,** 628-630.

Torchin, M. E. & Mitchell, C. E. 2004. Parasites, Pathogens, and Invasions by Plants and Animals. *Frontiers in Ecology and the Environment,* 2(4)**,** 183-190.

Tsakas, S. & Marmaras, V. 2010. Insect Immunity and Its Signaling: An Overview. *Invertebrate Survival Journal,* 7.

Tucker, M. R. 1994. Inter- and Intra-Seasonal Variation in Outbreak Distribution of the Armyworm, Spodoptera Exempta (Lepidoptera: Noctuidae), in Eastern Africa. *Bulletin of Entomological Research,* 84(2)**,** 275-287.

Tucker, M. R., Mwandoto, S. & Pedgley, D. E. 1982. Further Evidence for Windborne Movement of Armyworm Moths, Spodoptera Exempta, in East Africa. *Ecological Entomology,* 7(4)**,** 463-473.

United Nations 2020. The Sustainable Development Goals Report 2020. New York, USA.

Uzayisenga, B., Waweru, B., Kajuga, J., Karangwa, P., Uwumukiza, B., Edgington, S., Thompson, E., Offord, L., Cafa, G. & Buddie, A. 2018. First Record of the Fall Armyworm, Spodoptera Frugiperda (J.E. Smith, 1797) (Lepidoptera: Noctuidae), in Rwanda. *African Entomology,* 26**,** 244-246.

Valanne, S., Wang, J. & Ramet, M. 2011. The Drosophila Toll Signaling Pathway. *J. Immunol.*

Valentin, R. E., Fonseca, D. M., Nielsen, A. L., Leskey, T. C. & Lockwood, J. L. 2018. Early Detection of Invasive Exotic Insect Infestations Using Edna from Crop Surfaces. *Frontiers in Ecology and the Environment,* 16(5)**,** 265-270.

Van Lenteren, J. C., Bolckmans, K., Köhl, J., Ravensberg, W. J. & Urbaneja, A. 2018. Biological Control Using Invertebrates and Microorganisms: Plenty of New Opportunities. *BioControl,* 63(1)**,** 39-59.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. & Speleman, F. 2002. Accurate Normalization of Real-Time Quantitative Rt-Pcr Data by Geometric Averaging of Multiple Internal Control Genes. *Genome Biology,* 3(7)**,** research0034.1.

Vilaplana, L., Redman, E., Wilson, K. & Cory, J. 2008. Density-Related Variation in Vertical Transmission of a Virus in the African Armyworm. *Oecologia,* 155**,** 237-46.

Vilaplana, L., Wilson, K., Redman, E. & Cory, J. 2010. Pathogen Persistence in Migratory Insects: High Levels of Vertically-Transmitted Virus Infection in Field Populations of the African Armyworm. *Evolutionary Ecology,* 24(1)**,** 147-160.

Vilcinskas, A. 2019. Pathogens Associated with Invasive or Introduced Insects Threaten the Health and Diversity of Native Species. *Current Opinion in Insect Science,* 33**,** 43-48.

Vilcinskas, A., Stoecker, K., Schmidtberg, H., Röhrich, C. & Vogel, H. 2013. Invasive Harlequin Ladybird Carries Biological Weapons against Native Competitors. *Science (New York, N.Y.),* 340**,** 862-863.

Virgen, O. E., Campos, J. C., Bermudez, A. R., Velasco, C. R., Cazola, C. C., Aquino, N. I. & Cancino, E. R. 2013. Parasitoids and Entomopathogens of the Fall Armyworm

Spodoptera Frugiperda (Lepidoptera: Noctuidae) in Nayarit, Mexico. *Southwestern Entomologist,* 38(2)**,** 339-344.

Visalakshi, M., Varma, P. K., Sekhar, V. C., Bharathalaxmi, M., Manisha, B. L. & Upendhar, S. 2020. Studies on Mycosis of Metarhizium (Nomuraea) Rileyi on Spodoptera Frugiperda Infesting Maize in Andhra Pradesh, India. *Egyptian Journal of Biological Pest Control,* 30(1)**,** 135.

Vitousek, P., Dantonio, C. M., Loope, L., Rejmanek, M. & Westbrooks, R. 1997. Introduced Species: A Significant Component of Human-Caused Global Change. *New Zeland Journal of Ecology,* 21**,** 1-16.

Vodovotz, Y., Zamora, R., Lieber, M. J. & Luckhart, S. 2004. Cross-Talk between Nitric Oxide and Transforming Growth Factor-B1 in Malaria. *Current molecular medicine,* 4(7)**,** 787-797.

Vogel, S. 1966. Flight in Drosophila I. Flight Performance of Tethered Flies. *Journal of Experimental Biology,* 44.

Voigt, C. C., Fritze, M., Lindecke, O., Costantini, D., Pētersons, G. & Czirják, G. Á. 2020. The Immune Response of Bats Differs between Pre-Migration and Migration Seasons. *Scientific Reports,* 10(1)**,** 17384.

Wan, G., Hayden, A. N., Iiams, S. E. & Merlin, C. 2021. Cryptochrome 1 Mediates Light-Dependent Inclination Magnetosensing in Monarch Butterflies. *Nature Communications,* 12(1)**,** 771.

Wan Mohammad, W. N., Soh, L.-S., Wan Ismail, W. N. & Veera Singham, G. 2020. Infestation Pattern and Population Dynamics of the Tropical Bed Bug, Cimex Hemipterus (F.) (Hemiptera: Cimicidae) Based on Novel Microsatellites and Mtdna Markers. *Insects,* 11(8).

Wang, S., Minter, M., Homem, R. A., Michaelson, L. V., Venthur, H., Lim, K. S., Withers, A., Xi, J., Jones, C. M. & Zhou, J.-J. 2020. Odorant Binding Proteins Promote Flight Activity in the Migratory Insect, Helicoverpa Armigera. *Molecular Ecology,* 29(19)**,** 3795-3808.

Wang, X., Fuchs, J. F., Infanger, L.-C., Rocheleau, T. A., Hillyer, J. F., Chen, C.-C. & Christensen, B. M. 2005. Mosquito Innate Immunity: Involvement of Beta 1,3-Glucan Recognition Protein in Melanotic Encapsulation Immune Responses in Armigeres Subalbatus. *Molecular and biochemical parasitology,* 139(1)**,** 65-73.

Wang, Y. & Zhang, X. 2001. Studies on the Migratory Behaviours of Oriental Armyworm, Mythimna Separata (Walker). *Acta Ecologica Sinica,* 21(5)**,** 772-779.

Webster, K. A., Schach, U., Ordaz, A., Steinfeld, J. S., Draper, B. W. & Siegfried, K. R. 2017. Dmrt1 Is Necessary for Male Sexual Development in Zebrafish. *Developmental Biology,* 422(1)**,** 33-46.

Wegener, G. 1996. Flying Insects: Model Systems in Exercise Physiology. *Experientia,* 52(5)**,** 404-412.

Weir, B. S. 1996. *Genetic Data Analysis Ii: Methods for Discrete Population Genetic Data*: Sinauer.

Wennmann, J. T., Tepa-Yotto, G. T., Jehle, J. A. & Goergen, G. 2021. Genome Sequence of a Spodoptera Frugiperda Multiple Nucleopolyhedrovirus Isolated from Fall Armyworm (*Spodoptera Frugiperda*) in Nigeria, West Africa. *Microbiology Resource Announcements,* 10(34).

Westbrook, J., Nagoshi, R., Meagher, R., Fleischer, S. & Jairam, S. 2016. Modeling Seasonal Migration of Fall Armyworm Moths. *International Journal of Biometeorology,* 60(2)**,** 255-267.

Wikelski, M., Moskowitz, D., Adelman, J. S., Cochran, J., Wilcove, D. S. & May, M. L. 2006. Simple Rules Guide Dragonfly Migration. *Biology Letters,* 2(3)**,** 325-329.

Williams, T., Virto, C., Murillo, R. & Caballero, P. 2017. Covert Infection of Insects by Baculoviruses. *Frontiers in Microbiology,* 8(1337).

Wilson, K. 2021. *News, Updates from the Armyworm Network* [Online]. Lancaster, UK. Available: www.armyworm.org/ [Accessed 09/10/2020 2020].
Wilson, K. & Cotter, S. C. 2013. Chapter Three - Host–Parasite Interactions and the Evolution of Immune Defense. *In:* BROCKMANN, H. J., ROPER, T. J., NAGUIB, M., MITANI, J. C., SIMMONS, L. W. & BARRETT, L. (eds.) *Advances in the Study of Behavior.* Academic Press.
Wilson, K., Cotter, S. C., Reeson, A. F. & Pell, J. K. 2001. Melanism and Disease Resistance in Insects. *Ecology Letters,* 4(6)**,** 637-649.
Wilson, K. & Gatehouse, A. G. 1992. Migration and Genetics of Pre-Reproductive Period in the Moth, Spodoptera Exempta (African Armyworm). *Heredity,* 69(3)**,** 255-262.
Wilson, K. & Gatehouse, A. G. 1993. Seasonal and Geographical Variation in the Migratory Potential of Outbreak Populations of the African Armyworm Moth, Spodoptera Exempta. *Journal of Animal Ecology,* 62(1)**,** 169-181.
Wilson, K. & Reeson Andrew, F. 2002. Density-Dependent Prophylaxis: Evidence from Lepidoptera–Baculovirus Interactions? *Ecological Entomology,* 23(1)**,** 100-101.
Winter, D. 2012. Mmod: An R Library for the Calculation of Population Differentiation Statistics. *Molecular Ecology Resources,* 12.
Winterhalter, W. E. & Fedorka, K. M. 2009. Sex-Specific Variation in the Emphasis, Inducibility and Timing of the Post-Mating Immune Response in Drosophila Melanogaster. *Proceedings. Biological sciences,* 276(1659)**,** 1109-1117.
Withers, A. J., De Boer, J., Chipabika, G., Zhang, L., Smith, J. A., Jones, C. M. & Wilson, K. 2021. Microsatellites Reveal That Genetic Mixing Commonly Occurs between Invasive Fall Armyworm Populations in Africa. *Scientific Reports,* 11(1)**,** 20757.
Woestmann, L., Kvist, J. & Saastamoinen, M. 2016. Fight or Flight? – Flight Increases Immune Gene Expression but Does Not Help to Fight an Infection. *Journal of Evolutionary Biology,* 30(3)**,** 501-511.
Woodrow, K. P., Gatehouse, A. G. & Davies, D. A. 1987. The Effect of Larval Phase on Flight Performance of African Armyworm Moths, *Spodoptera Exempta* (Walker) (Lepidoptera: Noctuidae). *Bull. Entomol. Res.,* 77(1)**,** 113-122.
Woon Shin, S., Park, S.-S., Park, D.-S., Gwang Kim, M., Sun Chang, K., Brey, P. T. & Park, H.-Y. 1998. Isolation and Characterization of Immune-Related Genes from the Fall Webworm, Hyphantria Cunea, Using Pcr-Based Differential Display and Subtractive Cloning. *Insect Biochemistry and Molecular Biology,* 28(11)**,** 827-837.
Xi, Z., Ramirez, J. L. & Dimopoulos, G. 2008. The Aedes Aegypti Toll Pathway Controls Dengue Virus Infection. *PLOS Pathogens,* 4(7)**,** e1000098.
Xiao, H., Sandaltzopoulos, R., Wang, H.-M., Hamiche, A., Ranallo, R., Lee, K.-M., Fu, D. & Wu, C. 2001. Dual Functions of Largest Nurf Subunit Nurf301 in Nucleosome Sliding and Transcription Factor Interactions. *Molecular Cell,* 8(3)**,** 531-543.
Xiao, Y., Dai, Q., Hu, R., Pacheco, S., Yang, Y., Liang, G., Soberón, M., Bravo, A., Liu, K. & Wu, K. 2017. A Single Point Mutation Resulting in Cadherin Mislocalization Underpins Resistance against Bacillus Thuringiensis Toxin in Cotton Bollworm*. *Journal of Biological Chemistry,* 292(7)**,** 2933-2943.
Xing, L., Yuan, C., Wang, M., Lin, Z., Shen, B., Hu, Z. & Zou, Z. 2017. Dynamics of the Interaction between Cotton Bollworm Helicoverpa Armigera and Nucleopolyhedrovirus as Revealed by Integrated Transcriptomic and Proteomic Analyses*. *Molecular & Cellular Proteomics,* 16(6)**,** 1009-1028.
Yainna, S., Nègre, N., Silvie, P. J., Brévault, T., Tay, W. T., Gordon, K., Dalençon, E., Walsh, T. & Nam, K. 2021. Geographic Monitoring of Insecticide Resistance Mutations in Native and Invasive Populations of the Fall Armyworm. *Insects,* 12(5).
Yang, C.-C., Yu, Y.-C., Valles, S. M., Oi, D. H., Chen, Y.-C., Shoemaker, D., Wu, W.-J. & Shih, C.-J. 2010. Loss of Microbial (Pathogen) Infections Associated with Recent Invasions of the Red Imported Fire Ant Solenopsis Invicta. *Biological Invasions,* 12(9)**,** 3307-3318.

Yang, F., Hu, G., Shi, J. J. & Zhai, B. P. 2014. Effects of Larval Density and Food Stress on Life-History Traits of Cnaphalocrocis Medinalis (Lepidoptera: Pyralidae). *Journal of Applied Entomology,* 139(5)**,** 370-380.

Zacarias, D. A. 2020. Global Bioclimatic Suitability for the Fall Armyworm, Spodoptera Frugiperda (Lepidoptera: Noctuidae), and Potential Co-Occurrence with Major Host Crops under Climate Change Scenarios. *Climatic Change,* 161(4)**,** 555-566.

Zaghloul, H. A. H., Hice, R., Bideshi, D. K., Arensburger, P. & Federici, B. A. 2020. Mitochondrial and Innate Immunity Transcriptomes from <Span Class="Named-Content Genus-Species" Id="Named-Content-1">Spodoptera Frugiperda</Span> Larvae Infected with the Spodoptera Frugiperda Ascovirus. *Journal of Virology,* 94(9)**,** e01985-19.

Zambon, R. A., Nandakumar, M., Vakharia, V. N. & Wu, L. P. 2005. The Toll Pathway Is Important for an Antiviral Response in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America,* 102(20)**,** 7257.

Zamora-Aviles, N., Alonso-Vargas, J., Pineda, S., Isaac-Figueroa, J., Lobit, P. & Martinez-Castillo, A. M. 2013. Effects of a Nucleopolyhedrovirus in Mixtures with Azadirachtin on Spodoptera Frugiperda (J. E. Smith) (Lepidoptera: Noctuidae) Larvae and Viral Occlusion Body Production. *Biocontrol Science and Technology,* 23(5)**,** 521-534.

Zannou, I. D., Hanna, R., Agboton, B., De Moraes, G. J., Kreiter, S., Phiri, G. & Jone, A. 2007. Native Phytoseiid Mites as Indicators of Non-Target Effects of the Introduction of Typhlodromalus Aripo for the Biological Control of Cassava Green Mite in Africa. *Biological Control,* 41(2)**,** 190-198.

Zera, A. & Mole, S. 1994. The Physiological Costs of Flight Capability in Wing-Dimorphic Crickets. *Researches on Population Ecology,* 36(2)**,** 151-156.

Zhan, S., Merlin, C., Boore, Jeffrey l. & Reppert, Steven m. 2011. The Monarch Butterfly Genome Yields Insights into Long-Distance Migration. *Cell,* 147(5)**,** 1171-1185.

Zhang, K., Pan, G., Zhao, Y., Hao, X., Li, C., Shen, L., Zhang, R., Su, J. & Cui, H. 2017. A Novel Immune-Related Gene Hdd1 of Silkworm Bombyx Mori Is Involved in Bacterial Response. *Molecular Immunology,* 88**,** 106-115.

Zhang, L., Liu, B., Zheng, W., Liu, C., Zhang, D., Zhao, S., Li, Z., Xu, P., Wilson, K., Withers, A., Jones, C. M., Smith, J. A., Chipabika, G., Kachigamba, D. L., Nam, K., D'alençon, E., Liu, B., Liang, X., Jin, M., Wu, C., Chakrabarty, S., Yang, X., Jiang, Y., Liu, J., Liu, X., Quan, W., Wang, G., Fan, W., Qian, W., Wu, K. & Xiao, Y. 2020. Genetic Structure and Insecticide Resistance Characteristics of Fall Armyworm Populations Invading China. *Molecular ecology resources,* 20(6)**,** 1682-1696.

Zhang, L., Zhao, W., Wang, F. & Qin, D. 2019. Genetic Diversity and Population Structure of Natural Lycorma Delicatula (White) (Hemiptera: Fulgoridea) Populations in China as Revealed by Microsatellite and Mitochondrial Markers. *Insects,* 10(10).

Zhang, T., He, K. & Wang, Z. 2016. Transcriptome Comparison Analysis of Ostrinia Furnacalis in Four Developmental Stages. *Scientific Reports,* 6**,** 35008.

Zhang, W. & Swinton, S. 2009. Incorporating Natural Enemies in an Economic Threshold for Dynamically Optimal Pest Management. *Ecological Modelling - ECOL MODEL,* 220**,** 1315-1324.

Zhao, X. C., Feng, H. Q., Wu, B., Wu, X. F., Liu, Z. F., Wu, K. M. & Mcneil, J. N. 2009. Does the Onset of Sexual Maturation Terminate the Expression of Migratory Behaviour in Moths? A Study of the Oriental Armyworm, *Mythimna Separata*. *Journal of Insect Physiology,* 55(11)**,** 1039-1043.

Zheng, W., Li, Z., Zhao, J., Zhang, Y., Wang, C., Lu, X. & Sun, F. 2016. Study of the Long-Distance Migration of Small Brown Planthoppers Laodelphax Striatellus in China Using Next-Generation Sequencing. *Pest Management Science,* 72(2)**,** 298-305.

Zhou, Y.-M., Xie, W., Ye, J.-Q., Zhang, T., Li, D.-Y., Zhi, J.-R. & Zou, X. 2020. New Potential Strains for Controlling Spodoptera Frugiperda in China: Cordyceps Cateniannulata and Metarhizium Rileyi. *BioControl (Dordrecht, Netherlands)*.

Zhu, H., Casselman, A. & Reppert, S. M. 2008a. Chasing Migration Genes: A Brain Expressed Sequence Tag Resource for Summer and Migratory Monarch Butterflies (*Danaus Plexippus*) (Monarch Butterfly Est Resource). *PLoS ONE,* 3(1)**,** e1345.

Zhu, H., Gegear, R. J., Casselman, A., Kanginakudru, S. & Reppert, S. M. 2009. Defining Behavioral and Molecular Differences between Summer and Migratory Monarch Butterflies. *BMC Biology,* 7**,** 1-14.

Zhu, H., Sauman, I., Yuan, Q., Casselman, A., Emery-Le, M., Emery, P. & Reppert, S. M. 2008b. Cryptochromes Define a Novel Circadian Clock Mechanism in Monarch Butterflies That May Underlie Sun Compass Navigation. *PLOS Biology,* 6(1)**,** e4.

Zhu, X., Yuan, M., Shakeel, M., Zhang, Y., Wang, S., Wang, X., Zhan, S., Kang, T. & Li, J. 2014. Selection and Evaluation of Reference Genes for Expression Analysis Using Qrt-Pcr in the Beet Armyworm Spodoptera Exigua (Hübner) (Lepidoptera: Noctuidae). *PLOS ONE,* 9(1)**,** e84730.

Zou, Z., Lopez, D. L., Kanost, M. R., Evans, J. D. & Jiang, H. 2006. Comparative Analysis of Serine Protease-Related Genes in the Honey Bee Genome: Possible Involvement in Embryonic Development and Innate Immunity. *Insect Molecular Biology,* 15(5)**,** 603-614.

scientific reports

Check for updates

OPEN

Microsatellites reveal that genetic mixing commonly occurs between invasive fall armyworm populations in Africa

Amy J. Withers[1,2]✉, Jolanda de Boer[3], Gilson Chipabika[4], Lei Zhang[5], Judith A. Smith[3], Christopher M. Jones[6,7] & Kenneth Wilson[1,5]✉

Understanding the population structure and movements of the invasive fall armyworm (FAW, *Spodoptera frugiperda*) is important as it can help mitigate crop damage, and highlight areas at risk of outbreaks or evolving insecticide resistance. Determining population structure in invasive FAW has been a challenge due to genetic mutations affecting the markers traditionally used for strain and haplotype identification; *mitochondrial cytochrome oxidase I* (COIB) and the Z-chromosome-linked *Triosephosphate isomerase* (Tpi). Here, we compare the results from COIB and Tpi markers with highly variable repeat regions (microsatellites) to improve our understanding of FAW population structure in Africa. There was very limited genetic diversity using the COIB marker, whereas using the TpiI4 marker there was greater diversity that showed very little evidence of genetic structuring between FAW populations across Africa. There was greater genetic diversity identified using microsatellites, and this revealed a largely panmictic population of FAW alongside some evidence of genetic structuring between countries. It is hypothesised here that FAW are using long-distance flight and prevailing winds to frequently move throughout Africa leading to population mixing. These approaches combined provide important evidence that genetic mixing between invasive FAW populations may be more common than previously reported.

The fall armyworm (FAW, *Spodoptera frugiperda*) is a highly invasive crop pest in Africa, Asia and Australasia[1]. It is native to North America where it is largely migratory, surviving winters in southern Florida and Texas before migrating north as the temperature warms, though there is some evidence that parts of Central and South America, such as Puerto Rico, have more resident populations that rarely interact with FAW from elsewhere in America[2–4]. The migratory nature of FAW means that it has a strong flight ability, and some individuals can disperse as far as 300 miles before oviposition[5]. Wherever it disperses to, the effects are devastating, causing millions of tonnes of crops to be lost, resulting in huge economic losses as well as food shortages[6].

Understanding the migratory routes of FAW is important as these can be used to predict areas at risk and give farmers warning for early intervention techniques[2,7]. Additionally, understanding gene flow can help to predict outbreaks and foresee the spread of insecticide resistance that primarily occurs through the mixing of populations, leading to resistance alleles becoming more common in populations that were previously susceptible[7,8].

There is currently a lot known about FAW population structure and movements in its native range (North, Central and South America), enabling farmers to deal with outbreaks and minimise crop losses[2,7,9,10]. Much less is known about potential migration and population mixing in Africa, and much of the available research has been based on *mitochondrial cytochrome oxidase I* (COIB) and the Z-chromosome-linked *Triosephosphate isomerase* (Tpi) haplotypes[9,11,12]. There are two Tpi markers used for FAW, TpiE4 that is based on variation in exon 4 which can differentiate between the corn and rice strains, and TpiI4 that is based on intronic variation and has six recorded haplotypes (five corn, one rice) that can differentiate between strains and populations[9,11,12]. However, there is some disagreement between COIB and TpiE4 haplotypes in FAW in Africa for strain identification, with evidence suggesting that the COIB haplotypes are less reliable in distinguishing between invasive populations

[1]Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK. [2]Rothamsted Research, Harpenden AL5 2JQ, UK. [3]University of Central Lancashire, Preston PR1 2HE, UK. [4]Zambia Agriculture Research Institute, Chilanga, Zambia. [5]Chinese Academy of Agricultural Sciences, Shenzhen, China. [6]Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK. [7]Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Blantyre, Malawi. ✉email: a.withers@lancaster.ac.uk; ken.wilson@lancaster.ac.uk

across Africa and Asia[9,11,13]. This disagreement means that most individuals are identified as the rice strain with the COIB marker, and the same individuals are then identified as the corn strain with the TpiE4 marker. Given that the majority of samples have been collected on maize plants it has been suggested that the most accurate marker is likely to be the TpiE4 marker[9,11,13]. However, this confusion with the COIB and TpiE4 markers is most likely due to the hybridization of the corn and rice strains which has occurred in the invasive populations since FAW left its native range[14]. Furthermore, the COIB and Tpi markers show very little variation in the invasive FAW populations, for example, only two COIB haplotypes (COIB-RS, CSh4) and four TpiI4 haplotypes (TpiCa1a, TpiCa2a, TpiCa2b, TpiRa1) were previously reported in South Africa and India[12,13].

There is also strong evidence that the invasive populations in Asia are originally from Africa, with FAW from both continents showing similar haplotype frequencies and the same mutation affecting the COIB strain identification marker[11–13]. Understanding the source of the FAW population via population genetics approaches is, therefore, an important area of research in curbing further spread, and this study addresses this by using microsatellites to determine population structure and genetic diversity across Africa.

Microsatellites are highly variable, repeat regions of DNA that are useful when studying genetic mixing within insect populations at continental scales. For example, in two hoverfly species (*Episyrphus balteatus* and *Sphaerophoria scripta*) microsatellites revealed high levels of genetic mixing, suggesting frequent migratory movements across Europe over a very large geographical scale, predominately occurring along the North–South axis[15]. Additionally, microsatellites can also be used to detect genetic differentiation at much smaller scales, such as between reduviid bugs (*Triatoma dimidiate*) in neighbouring villages in Guatemala[16].

Microsatellites have previously been identified in FAW using populations from Texas, Mississippi, Puerto Rico and Brazil[17,18]. These microsatellites were variable enough to distinguish between three genetically distinct populations and were able to identify migrants between those populations[18]. Therefore, considering the limited variability and confusion around the COIB and Tpi haplotypes, FAW microsatellites might be a better way to identify population structure in FAW across Africa.

To improve current understanding of population movements of FAW in Africa, in this study we explored population genetic structure across FAW larvae collected from six African countries (Malawi, Rwanda, Kenya, Sudan, Kenya and Ghana) between 2017 and 2019. We used traditional strain and haplotyping methods for FAW (COIB, TpiE4 and TpiI4), as well as eight highly variable FAW microsatellite loci to determine genetic structure and mixing across countries.

Results

Strain identification and haplotyping using COIB and TpiE4 markers. The COIB marker was analysed using an enzyme based PCR assay[19], and the TpiE4/TpiI4 product was sequenced using Sanger sequencing. The expected strain discordance between the COIB and TpiE4 markers was observed in all countries, with the markers only reporting the same strain in 19% of samples (see Supplementary Table S1 online). In all countries, both markers identified larvae of the corn and rice strain (Fig. 1). Overall, the COIB marker most frequently reported samples as the rice strain (mean ± S.E. = 72% ± 0.09), whereas the TpiE4 marker reported them as the corn strain (mean ± S.E. = 92% ± 0.02). Both markers showed very similar strain frequencies across Malawi, Rwanda, Sudan and Zambia. In Ghana, more samples were reported as corn strain (63%) than rice using the COIB marker compared to the other countries, and there was significant variation in the distribution of the corn strain based on the COIB marker ($\chi_4 = 53.17$, $P < 0.001$). However, when using the TpiE4 marker, the proportion reported as the corn strain was similar across all five countries ($\chi_4 = 1.16$, $P = 0.885$). Those larvae identified as corn strain by the COIB marker in Ghana (N = 45), Rwanda (N = 16), Sudan (N = 6) and Zambia (N = 8) were sequenced using Sanger sequencing to determine the haplotype. All larvae were identified as CSh4 suggesting very little genetic differentiation based on COIB haplotypes.

In all countries, the intronic TpiI4 marker identified both corn and rice strain FAW, with the corn strain (82–99%) being more common compared to the rice strain (1–18%) (Fig. 1A). The most common haplotype in every country was TpiCa1a, and the rarest was TpiCa2C (Fig. 1B). A novel rice haplotype (TpiRa1b) was identified in samples from Malawi, Rwanda and Sudan, where no larvae were of the previously recorded rice haplotype (Fig. 1B,C). The greatest number of different haplotypes was observed in Ghana, with four different haplotypes identified (TpiCa1a, TpiCa2a, TpiCa2C, TpiRa1a). Heterozygotes were recorded in all countries, however due to ambiguity in which haplotype combinations these were, they were only identified as heterozygotes (Fig. 1B). An amova was carried out on the TpiI4 alignment based on genetic distances and showed significant differences between the six countries, however the total variance explained by differences between countries was low, with most of the genetic variation being between individuals within countries, which would suggest a largely panmictic population (Table 1). To further check for genetic structuring based on TpiI4 markers, a PCA was carried out using the genetic distance between sequences and this showed clustering based on strain identification, but no evidence of structuring between the six countries (Supplementary Fig. S1A online).

Microsatellite locus information. Microsatellites were amplified by PCR individually, and then genotyped on a ABI3500 sequencer. All eight microsatellites successfully amplified, and the number of alleles found ranged from 3 to 13 (Table 2). Twenty-one individuals (23%) had missing allele data, this ranged from 1 to 3 loci per individual, with an average of 0.34 (Table 2). Null allele frequencies were high for four alleles: Spf1502, Spf343, Spf997 and Spf670 (Table 2). Seven of the eight microsatellites significantly deviated from *Hardy–Weinberg equilibrium* (HWE) when all individuals were considered together (Table 2). However, some of these microsatellites were in HWE at the within-country level (see Supplementary Table S2 online). The *index of association* (rbarD) metric measures how likely individuals are to be the same at one particular locus in relation to other loci, and how this compares to other individuals, and can give a good indication of linkage between loci[20]. This

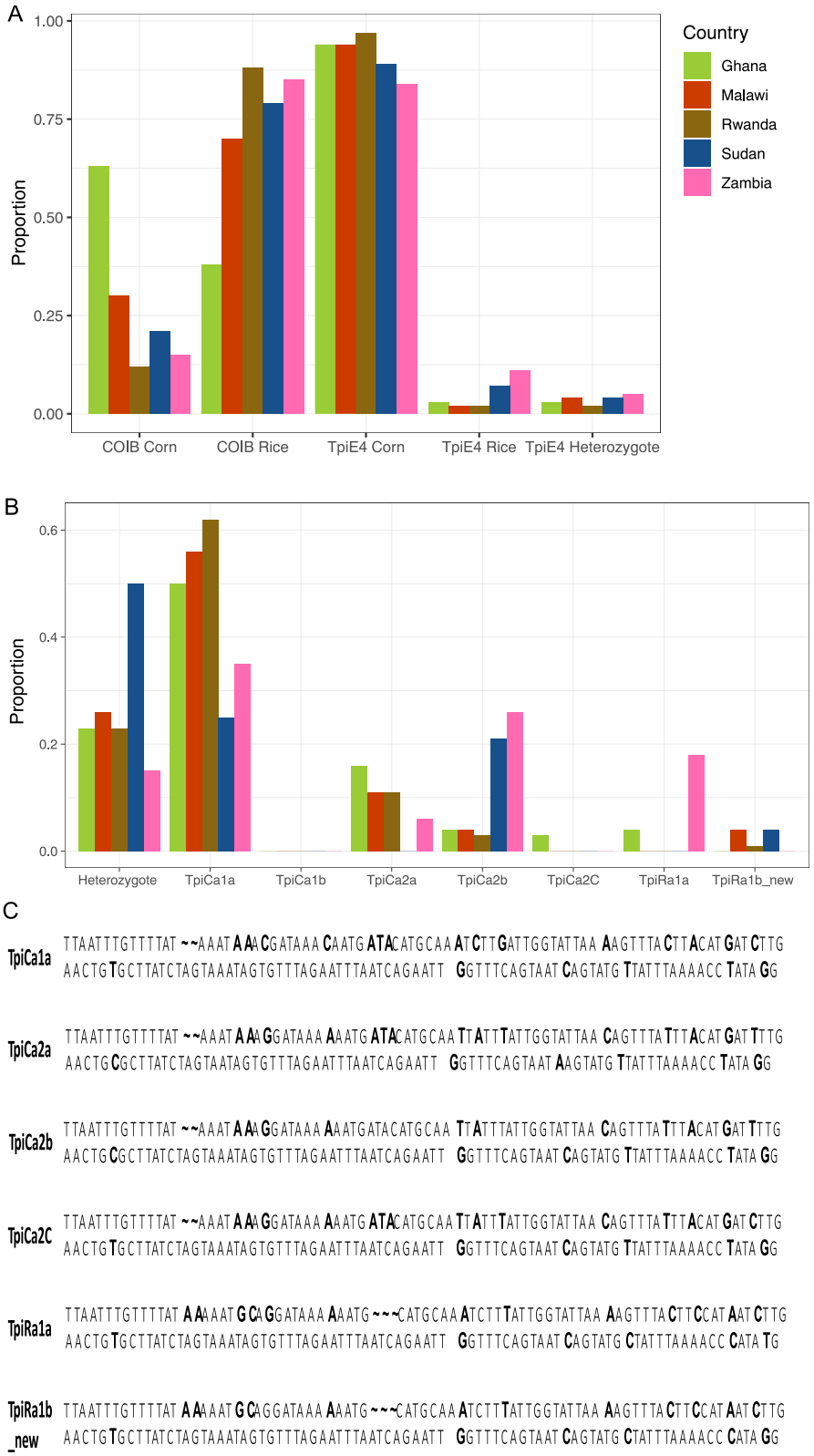


Figure 1. Strain and haplotype identification of FAW larvae using COIB, TpiE4 and TpiI4 markers. (**A**) Strain identification of FAW larvae using COIB and TpiE4 markers. The number of samples for COIB:TpiE4 markers tested per country are Ghana 72:72, Malawi 40:95, Rwanda 127:126, Sudan 28:28 and Zambia 53:44. (**B**) Proportion of each TpiI4 haplotype identified. (**C**) Sequences of each TpiI4 haplotype identified, variable positions are shown in bold. The number of samples for the TpiI4 marker for each country are Ghana N = 70, Malawi N = 27, Rwanda N = 141, Sudan N = 24 and Zambia N = 34.

Variation	Df	Sum of Squares	Variance components	Total variance (%)	*P* value
Between countries	4	13.40	0.04	3.51	0.002
Between individuals within countries	292	342.66	1.17	96.49	NA
Total	296	356.06	1.22	100	NA

Table 1. Results of an amova to analyse differences between the six countries based on TpiI4. *P* value was calculated using a randomization test with 999 permutations.

Locus	Individuals	Number of alleles	Number of individuals with missing data	Allele size range (bp)	Null allele frequency	Hardy–Weinberg equilibrium *P*
Spf1502	82	10	10	124–141	*0.62*	**< 0.001**
Spf789	86	13	5	182–199	0.11	**< 0.001**
Spf343	91	8	1	107–127	*0.35*	**< 0.001**
Spf997	90	7	2	79–113	*0.23*	**< 0.001**
Spf1706	91	3	1	118–126	0.16	**< 0.001**
Spf1592	87	11	5	187–217	0.00	**0.048**
Spf918	88	6	4	111–123	0.00	0.578
Spf670	90	7	2	128–152	*0.45*	**< 0.001**

Table 2. Locus and allele information for each of the eight microsatellites, and HWE results. Those loci with high null allele frequencies are in italics. Loci which significantly deviate from HWE are in bold and were calculated using a Monte Carlo Exact Test.

	Heterozygosity		Population differentiation			F-statistics			
Locus	*Hs*	*Ht*	*Nei's Gst*	*Hedrick's Gst*	*Jost's D*	*Fst*	*Fis*	*CI Fis (−)*	*CI Fis (+)*
Spf1502	0.76	0.82	0.07	0.36	0.30	0.05	0.75	0.19	0.66
Spf789	0.79	0.89	0.11	0.60	0.54	0.10	0.10	0.03	0.52
Spf343	0.74	0.75	0.01	0.05	0.04	0.00	0.52	0.19	0.61
Spf997	0.67	0.69	0.04	0.14	0.10	0.03	0.35	0.13	0.44
Spf1706	0.18	0.18	0.03	0.04	0.01	0.02	0.26	0.07	0.46
Spf1592	0.85	0.86	0.01	0.10	0.09	0.01	−0.01	0.06	0.54
Spf918	0.64	0.65	0.01	0.03	0.02	0.01	−0.02	0.19	0.66
Spf670	0.79	0.80	0.01	0.05	0.04	−0.01	0.63	0.03	0.52
All	NA	NA	0.04	0.14	0.03	0.03	0.33	NA	NA

Table 3. Genetic differentiation measures for FAW in Africa based on the eight microsatellites. In all three measures tested, a value of 0 suggests very little genetic differentiation (panmixia) and 1 suggests high levels of segregation. All measures are based on *Hs* (heterozygosity within populations) and *Ht* (heterozygosity without population structure). F-statistics represent genetic variance in a subpopulation compared to the whole (*Fst*—values closer to 1 suggest high levels of differentiation between populations) or in a subpopulation compared to individuals within that subpopulation (*Fis*—values close to 1 suggest high levels of inbreeding in populations). Negative values of *Fst* and *Fis* should be interpreted as 0 and suggest very low differentiation of populations (*Fst*) or very low chance of inbreeding (*Fis*). Confidence intervals of *Fis* based on bootstrapping are also provided.

metric was calculated and suggested a possible high chance of linkage between three pairs of loci (Spf1592 and Spf1502, Spf1592 and Spf997, Spf918 and Spf997) (see Supplementary Fig. S2 online). However, a composite linkage disequilibrium test, which measures the association between two alleles[21], did not find any significant evidence of linkage disequilibrium (see Supplementary Table S3 online).

Population differentiation based on microsatellites. Population differentiation can be measured in several ways; here we used three common measures (Nei's *Gst*, Hedrick's *Gst* and *Jost's D*) and each suggested that there was very little evidence of population differentiation across Africa[22–24] (Table 3). In all three measures tested, a value of 0 suggests little genetic differentiation (panmixia) and 1 suggests high levels of segregation. The range of the three measures across all loci was 0.03 to 0.14 (Table 3). There was also evidence of low genetic variance based on *Fst* between countries at each locus tested (Table 3). Pairwise *Fst* values between the six countries

Variation	Df	Sum of squares	Variance components	Total variance (%)	*P* value
Between countries	5	69.24	0.197	3.25	0.001
Between individuals within countries	86	672.08	1.961	32.41	0.001
Within individuals	92	358.17	3.893	64.34	0.001
Total	183	1099.49	6.051	100	NA

Table 4. Results of an amova to analyse differences between the six countries in this analysis based on the microsatellites. *P* value was calculated using a randomization test with 999 permutations.

ranged from − 0.02 to 0.08 (mean ± S. E. = 0.03 ± 0.01) suggesting high levels of population mixing (Supplementary Table S4 online). The level of inbreeding occurring within populations can be inferred from *Fis*, however, this varied between loci with high levels suggested for some loci (e.g. Spf1502 and Spf670), but low for others (e.g. Spf1592 and Spf789) (Table 3). These results suggest that in Africa, FAW may frequently mix with FAW from other countries suggesting that very little population differentiation is occurring.

Population differentiation was further analysed using an amova to determine if the genetic distance between individuals varies by country, location within country or sampling year (see Supplementary Table S5 online). There was no significant difference between samples from locations within countries ($F_{4,81} = 1.17$, $P = 0.120$), or between sampling years ($F_{1,81} = 0.96$, $P = 0.543$). The amova suggested, however, that FAW from each country were genetically different to FAW from other countries ($F_{5,74} = 1.86$, $P = 0.001$).

As *Country* was the only significant factor influencing FAW population differentiation, a second amova was carried out to determine genetic variation between and within countries. This suggested significant differences between the six countries, however the total variance explained by differences between countries was low, and most of the genetic variation was found within individuals which would suggest a largely panmictic population (Table 4). To further check for genetic structuring based on the microsatellite markers, a PCA was carried out using the genetic distance between individuals and this showed no evidence of structuring between the six countries (Supplementary Fig. S1B online).

Population clustering based on microsatellites. Clustering was carried out using an admixture model in *STRUCTURE*, with the number of clusters selected using *Delta K* (Evanno method) and *LnPr(K)* methods[25]. Based on *Delta K* there were three genetically distinct clusters in FAW (Fig. 2A,B). Based on *LnPr(K)*, the most likely number of clusters was five (Fig. 2C,D). In both 3 and 5 cluster scenarios, FAW from Sudan and Zambia were more genetically isolated from the four other countries, though some individuals from the other four countries do show similar assignment patterns suggesting population mixing does occur between all countries. Samples from Ghana, Kenya, Malawi and Rwanda appear very similar to each other, suggesting high levels of population mixing between these countries (Fig. 2C,D, clustering with two and four clusters is shown in Supplementary Fig. S3 online). Based on the similarities between the structuring results for 3 and 5 clusters, and that the *LnPr(K)* begins to plateau after K = 3 we propose that population structure of FAW in Africa is best described by three genetic clusters. To identify potential substructure in FAW from the four countries exhibiting evidence of genetic similarity (Ghana, Kenya, Malawi and Rwanda), a separate analysis was performed in *STRUCTURE*. This identified 3 genetic clusters as the most likely scenario, based on both *DeltaK* and *LnPr(K)*, and further confirmed high levels of mixing between the countries, with no strong evidence of substructure identified (Fig. 2E–G).

STRUCTURE has been shown to miss some subdivision when clustering individuals[26], therefore, population clustering analysis was also carried out by identifying clusters *de-novo* (i.e., with no prior population information provided) and then using *Discriminant Analysis Principal Components (DAPC)*. This approach determines the number of possible clusters by running successive K-means clustering, and selecting the most suitable cluster based on Bayesian Information Criterion and the number of PCs to keep was calculated to be 7 using the a-score[26]. This method identified three clusters as the best model based on BIC (BIC = 120.67) in FAW (Fig. 3A). Based on three clusters, FAW from Sudan were more genetically different to populations from elsewhere in Africa with no individuals assigned to cluster 3, whereas, cluster 3 individuals were found in all other countries (Fig. 3B–D). The three clusters highlighted similarities between the adjacent countries of Zambia and Malawi, with 50% and 44% of individuals respectively from these countries assigned to cluster 3, and similarities between Kenya and Rwanda, with 23% and 19% of individuals assigned to cluster 3 respectively (Fig. 3B–D). Ghana showed most similarities with Kenya and Rwanda, with 25% of individuals assigned to cluster 3 (Fig. 3B–D).

Discussion

This study is the first to use microsatellites to determine FAW population mixing and genetic diversity in Africa. Considering the limited genetic diversity and unreliability of the COIB and TpiE4 haplotypes for strain identification and the potential for confusion caused by corn and rice strain hybrids[11–14,27,28], we sought to quantify the degree of population structuring in FAW in Africa using a more robust microsatellite approach. This revealed that microsatellites had higher levels of genetic diversity compared to the COIB and TpiE4 markers, revealing that FAW in Africa is largely a panmictic population.

The previously reported discordance between the TpiE4 and COIB markers for strain identification was mirrored in this study, with very little agreement occurring between the markers. Furthermore, based on the COIB haplotypes it was not possible to determine genetic differentiation between the countries as only COIB CSh4

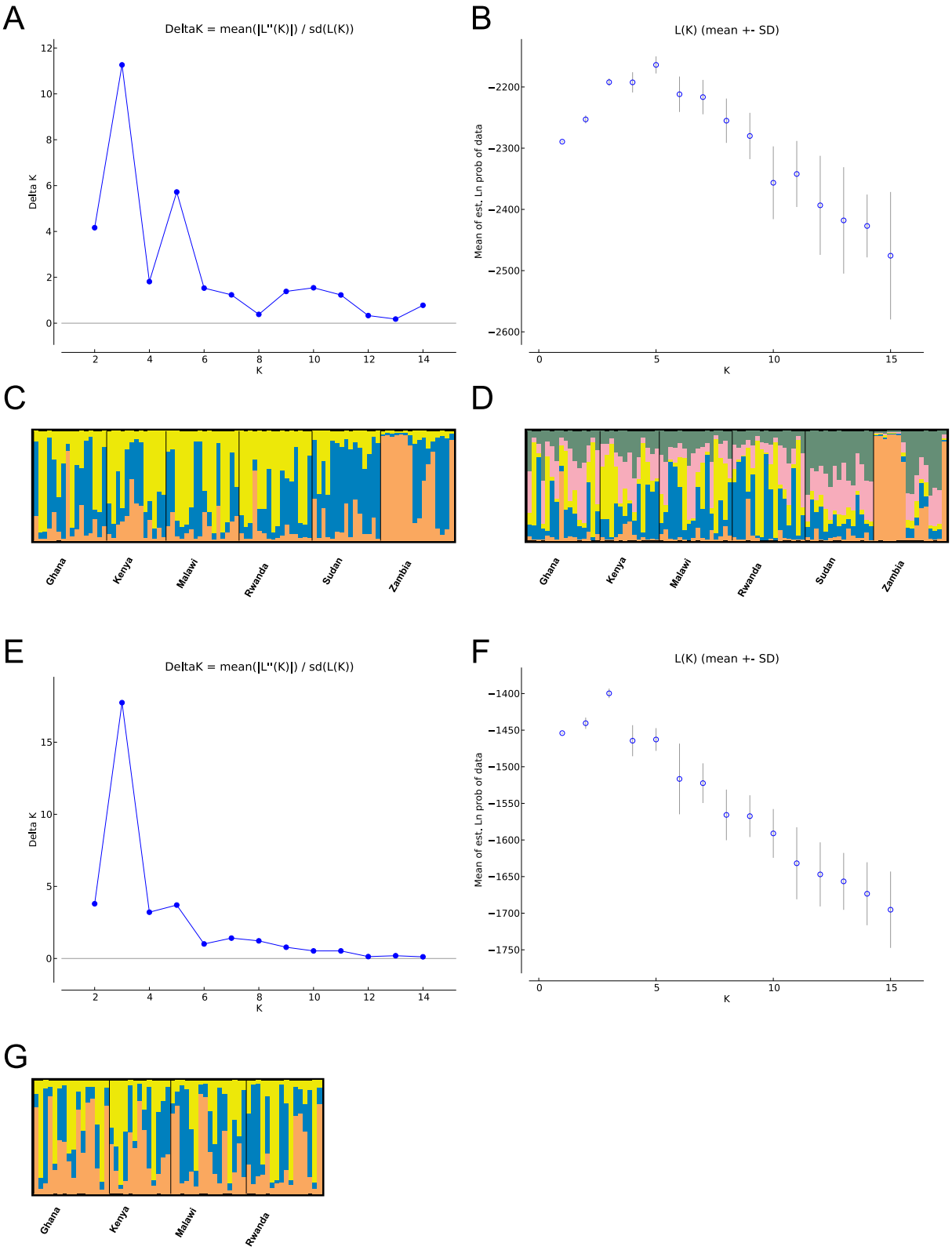


Figure 2. Genetic structure of FAW as assigned by *STRUCTURE* analysis of microsatellites. Panels (**A**) to (**D**) show the results of STRUCTURE with all six countries. Panel (**A**) shows the *DeltaK*, Panel (**B**) shows the *LnPr(K)* for each cluster. Panel C shows the admixture plot for the three genetic clusters based on *DeltaK*. Panel D shows the admixture plot for 5 genetic clusters based on *LnPr(K)*. Panels E to G show the results of STRU CTURE carried out to assess substructure hierarchically. Panel (**E**) and (**F**) show the *DeltaK* and *LnPr(K)* for each cluster respectively. Panel (**G**) shows the admixture plot for the three genetic clusters based on both *DeltaK* and *LnPr(K)*.

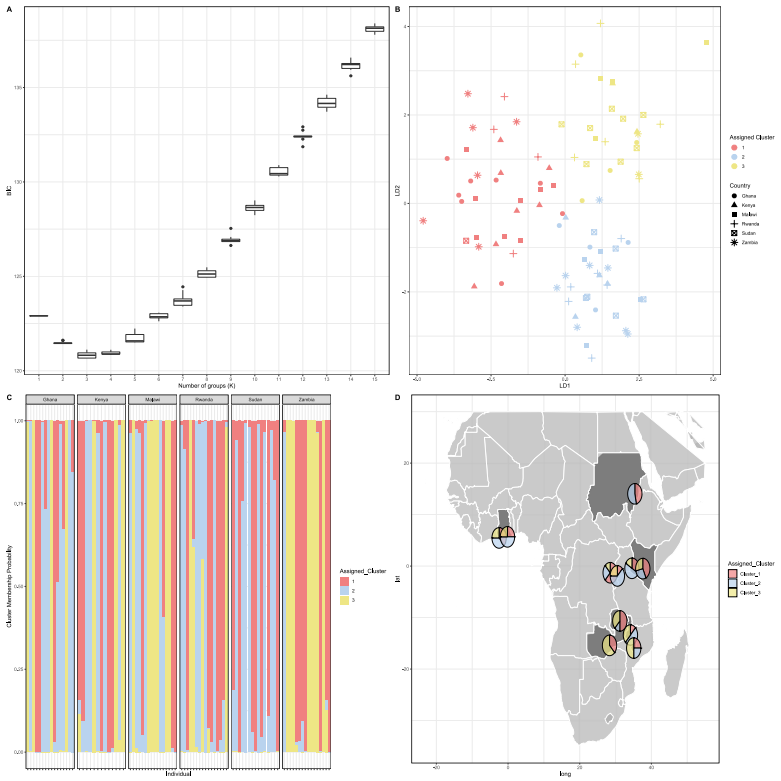


Figure 3. *DAPC* clustering (k = 3) and assignment of individuals from each country based on microsatellites. (**A**) The lowest BIC represents the best number of clusters, which here is 3. (**B**) The position of individuals on the first two principal components, and in (**C**) the membership probability of individuals to that cluster. (**D**) Assigned clusters for each sampling location across Africa.

was found. The intronic TpiI4 marker showed more variation between the individuals, however, the vast majority of larvae were TpiCa1a, which is in line with previous studies investigating FAW in Africa and Asia[12,13,28]. Previous work based on these markers in Africa concluded that there were significant differences between some African countries with widely separated populations being genetically distinct[28]. Whilst the findings here using the TpiI4 marker do support some evidence of genetic variation between countries, it was low, suggesting more of a panmictic population of FAW across Africa based on this marker.

The low genetic variability observed with the COIB marker, and both TpiE4 and TpiI4 markers, limit the analyses that can be carried out and reduce the likelihood of genetic differentiation between countries being detected. By using highly variable microsatellites, we were able to overcome this challenge to determine genetic differentiation between FAW from different countries in Africa, as well as some similarities, suggesting the possible presence of both resident and migratory populations of FAW throughout the continent.

Most of the microsatellites in this study were out of Hardy–Weinberg equilibrium (HWE), whereas in previous population genetics studies using microsatellites in FAW from Paraguay and Brazil, no loci were found to be out of HWE[8]. However, the deviation observed in the present study is to be expected in invasive FAW populations, which have been through a tight bottleneck, given that they probably originated from a small source population in Africa, providing further evidence of a common origin for FAW which then subsequently spread across the continent[9,27]. The microsatellites also showed evidence of a genetic bottleneck and loss of diversity in the African FAW compared to populations in Texas, Mississippi, Puerto Rico, and Brazil. For example, previously reported allele sizes for locus Spf997 were in the range of 95 to 139[8,17], whereas in this study the allele size range for the same locus was 79 to 113. The evidence of this genetic bottleneck throughout Africa offers more evidence of a single origin population instead of multiple introduction events. It is likely that if multiple incursion events had occurred then the microsatellite size ranges observed here would have matched more closely with those previously recorded.

Although FAW in Africa are likely to have undergone a population bottleneck at the time of invasion, the range of alleles for each locus identified in this study (3 to 13) was similar to that previously reported from Paraguay and Brazil (3 to 15)[8,18]. Based on this range of alleles, previous work found genetic differentiation between northern and southern FAW populations across Brazil and Paraguay, as well as gene flow across all populations sampled[8]. This indicates that despite a recent bottleneck there is still sufficient genetic diversity in microsatellite regions to enable population genetic studies of FAW in Africa.

Populations from the six countries (Kenya, Ghana, Malawi, Rwanda, Sudan and Zambia) did not show strong signs of population differentiation when using traditional measures (Nei's *GST*, Hedrick's *GST* and Jost's *D*). This

indicates that these populations mix frequently, and no strong genetic structure is evident. This was supported by the amova which showed that most of the genetic variance was occurring between individuals.

This lack of population differentiation between countries provides evidence consistent with FAW undergoing long distance migratory flights in Africa creating a panmixia of populations. Additionally, there was no evidence of genetic differentiation between samples from different sampling locations within the same country, confirming that populations are mixing within countries. This has significant consequences for the evolution and spread of insecticide resistance, as resistance alleles can spread rapidly throughout each country and across Africa. This is an important finding as insecticide resistance (organophosphate and pyrethroid resistance) has already been reported in FAW in China, so is highly likely to be present in Africa[14]. Considering the key role that long-distance, migratory flights played in the rapid spread of insecticide resistance both within and across continents in the invasive cotton bollworm (*Helicoverpa armigera*)[29–32], it is important to consider the implications of frequent, long-distance flights that seem to be occurring in FAW.

The evidence of panmixia contrasts with previous results based on the COIB and Tpi markers, which analysed FAW samples from across Africa and found evidence of genetic differentiation between geographically widespread countries[9,28]. Further investigation with microsatellites using clustering approaches show that whilst the countries included in this study are similar genetically (e.g. Kenya, Rwanda, and Ghana), others are more differentiated (e.g. Sudan). We conclude from this that genetic mixing of FAW populations is occurring widely across Africa, however, there are some FAW possibly forming resident and partially segregated populations, as seen in parts of South America and the Caribbean[7]. Alternatively, the possibility that FAW have not been in Africa long enough to evolve population differentiation should also be considered.

Previous reports based on COIB and Tpi suggested a possible east–west divide between FAW populations[9], or no clear pattern of division between populations[28]. Our study using microsatellites found that the two most genetically distinct populations are the most northerly and most southerly populations. African countries located further south (Zambia, Malawi) showed more similarities to each other compared with countries further north (Kenya, Rwanda, Ghana and Sudan) (e.g., fewer individuals were assigned to cluster 3 in the north compared to the south). This pattern of genetic separation coincides with the known migratory routes of the congeneric African armyworm (*Spodoptera exempta*) in eastern Africa, which follow the movement of the dominant winds each season, typically moving moths towards the north-west from Kenya and northern Tanzania, and a more south-westerly movement across southern Africa from Malawi[33,34]. This is also aligned with the movement of the inter-tropical convergence zone (ITCZ), with the wind direction (and hence seasonal migration) being broadly south-easterly north of the equator and north-easterly south of the equator[34]. Based on the high levels of mixing between FAW populations alongside this evidence of some genetic structuring between northern and southern populations, it is hypothesised that FAW may also follow the movement of the dominant winds if they are migratory in Africa as, like many other insects, they rely on wind to support high-altitude long-distance flights[2,5,35,36].

This study highlights the benefits of using multiple approaches to study genetic diversity, with evidence presented for both widespread genetic mixing between populations alongside some segregation between countries. This is most likely due to a proportion of FAW adults undergoing long-distance migratory flights whilst the remaining FAW form more sedentary, resident populations. These results provide important evidence that genetic mixing between FAW populations throughout Africa may be more common than previously reported. This has important consequences for FAW management when considering factors such as the spread of insecticide resistance and crop infestations across borders.

Methods

Sample collection. FAW larvae for sequencing with COIB:TpiI4:TpiE4 were collected from Ghana (N = 72:70:72, 2017), Malawi (N = 95:27:40, 2018 and 2019), Rwanda (N = 127:127:141, 2017), Sudan (N = 28:28:24, 2017) and Zambia (N = 44:53:34, 2017) and stored in ethanol. FAW larvae for microsatellite analysis were collected from Ghana (N = 16, 2017, maize), Kenya (N = 13, 2019, maize), Malawi (N = 8, 2018 and N = 8, 2019, maize), Rwanda (N = 16, 2017, maize), Sudan (N = 15, 2017, maize), and Zambia (N = 16, 2017, maize) and stored in ethanol. Full collection details are provided in Supplementary Table S1 Online for COIB and Tpi markers and Supplementary Table S5 Online for microsatellite markers.

DNA extraction. DNA was extracted from samples following the standard protocol for tissue in the Qiagen DNeasy Blood and Tissue kit. DNA was stored in buffer AE at – 20 °C. The protocol was altered slightly for extracting DNA from larvae collected in Sudan, these modifications were 200 µl ATL and an additional 200 µl 1 × SSC before incubation and the DNeasy Spin Column was centrifuged at 13,000 RPM.

Strain identification and haplotyping using COIB and Tpi markers. DNA was amplified for strain identification using COIB (F: 5′TACACGAGCATATTTTACATC, R: 5′GCTGGTGGTAAATTTTGATATC[27]) and TpiI4/TpiE4 (F: 5′ATGATTAGGACATCGGAGC, R:5′ATGTAATCCAGTCAATGCCTA[37], modified by de Boer). Cycling parameters for both COIB and TpiI4/TpiE4 were 94 °C 10 min, 33 cycles of 94 °C 1 min, 55 °C 1 min, 72 °C 1 min and then a final extension of 72 °C for 5 min. Following COIB amplification, the product was incubated at 37 °C for 2 h with 1 µl EcoRV restriction enzyme and 2 µl NEBuffer to determine FAW strain. EcoRV cuts the amplicon at position 1182 bp if the sample is from the rice strain resulting in two visible bands, and does not cut for the corn strain resulting in one larger band when the product is run on a gel electrophoresis (Table 3). There are five known haplotypes of the COIB marker, these are corn h1 ($A_{1164}A_{1287}$), corn h2 ($A_{1164}G_{1287}$), corn h3 ($G_{1164}A_{1287}$), corn h4 ($G_{1164}G_{1287}$) and rice ($T_{1164}A_{1287}$)[27] and unidirectional Sanger sequencing was used to verify the COIB corn haplotypes. Sequencing reactions contained 0.75 µL BigDye® Reaction Mix, 1.70 µL 5 × BigDye® Sequencing Buffer, 0.32 µL 10 µM Forward primer, 5–20 ng template DNA and H_2O to sup-

Name	GenBank identification	Simple sequence repeat (SSR)	Forward primer (5′–3′)	Reverse primer (5′–3′)
Spf343	HM752609	(TG)12	[6FAM]GTCAAAGTTTTA CATGGAAGCGTG	CCCATCTGTTTGTCCACA GTAAAG
Spf670	HM752637	(CAT)5	[6FAM]GGGAGAGGTTTC TAGCTTCTACGG	GAGGAGCCTTGGTTCAAT AGTGC
Spf789	HM752653	(CACAC)4	[6FAM]CGACACGTTGAT TGCTCACAG	AATCTTTTATCACAATTC GCAGCC
Spf918	HM752666	(TG)6	[6FAM]GCGAAATTGTTT TAATGTGGGTTG	ACGACCTATACGGACCTT GTTACG
Spf997	HM752675	(TACA)4	[6FAM]TTGATGCATGAA TTTTCAAACGAG	ATCACGTTGTGGTCCAAT CAATG
Spf1502	HM752731	(CA)12	[6FAM]TTTGCAATTTTAGTT ACAAACGTCCTC	TATTGATAGCCTCGTGTT TGACCC
Spf1592	HM752740	(TG)10	[6FAM]GGTTCCTGTTAT CACCTGCAGTA	CTATGTAGTTTATGTTAA TTCGCACGAT
Spf1706	HM752751	(AC)9	[6FAM]CCACTGTACTGTGAT AAACAGATGGC	ATGATCATACAAAGTGCA TCCGTG

Table 5. Microsatellite primer details.

plement the reaction to 10 µL. The sequencing reaction was preincubated for 1 min at 96° C followed by 25 cycles of: 10 s at 96° C; 5 s at 50° C; 4 min at 60° C. Excess incorporated dye-terminators were removed using EDTA/Ethanol precipitation before resuspending in 13 µL Hi-Di® formamide and capillary gel electrophoresis on an ABI 3500 Genetic Analyzer. Strain identification was carried out using the Tpi marker by Sangar sequencing following the same protocol as for COIB based on nucleotide variation in exon-4 (TpiE4), where the corn strain has base C_{183}, the R strain has base T_{183} and hybrids (males only) have C/T_{183} [27]. Tpi Intron 4 (TpiI4) was used to determine TpiI4 haplotypes based on 18 previously recorded highly variable positions[12,37]. For sequencing analysis, raw sequences were assembled and aligned using ClustalW in BioEdit[38,39]. Statistical analysis on strain and haplotype distributions for TpiE4 were carried out in R using a Poisson GLM followed by a Chi^2 test using the amova function. Based on the TpiI4 haplotypes, an amova was carried out using the POPPR package as this gave details of the variance explained within and between samples and populations (Kamvar et al. 2014), from which a *P* value was calculated using a randomization test with 999 permutations. The genetic distance computed for the amova was also used for Principal Components Analysis (PCA) using the *prcomp* function in R.

Microsatellite amplification. Eight highly variable microsatellites were selected for amplification based on them showing the greatest diversity in FAW in previous studies[8,17], the microsatellite primer details are shown in Table 5. Each sample was amplified in individual 20 µl reactions composed of 2 µl EasyTaq® Buffer, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer, 0.4 µl 10 mM dNTPs, 0.1 µl EasyTaq® DNA Polymerase, 13.5 µl H_2O and 2 µl DNA. Amplification conditions were 95 °C for 1 min, 30 cycles of 95 °C 30 s, 60 °C 30 s, 68 °C s and a final extension of 68 °C for 5 min. Once amplified, samples were stored at 20 °C until ready for genotyping.

Microsatellite genotyping. Fragment genotyping was carried out on an ABI3500 sequencer. Each reaction was composed of 11 µl HiDi Formamide, 0.4 µl Rox500 size standard and 1 µl PCR product (Spf343, Spf997 and Spf1706) or 0.5 µl PCR product (Spf1592, Spf670, Spf789, Spf918, Spf1502). Genotyping results were viewed on Thermo Fisher Connect™. The threshold for successful amplification was > 100RFU, and for heterozygotes the minor peak was > 50% of the major peak. Alleles were called based on size measurements and peaks determined to be the same allele if size measurements were within 0.5 nucleotides of each other (for example, a size of 150.2 and 150.6 were both classed as 150).

Microsatellite analysis. Samples with fewer than 5 microsatellites amplified were removed from the analysis. Microsatellite analysis was carried out in R (v. 4.0.3)[40]. Hardy–Weinberg equilibrium was tested using the PEGAS R package[41]. The frequency of null alleles was determined using the Chakraborty et al. (1994) formula through the POPGENREPORT R package[42]. Heterozygosity and F-statistics were calculated using the HIERFSTAT package[43]. Genetic differentiation was measured using the MMOD package (G_{st} and Jost's D)[44]. Linkage disequilibrium was calculated using an association index using the POPPR package[45] and by composite linkage disequilbrium using GenePop (v 4.7)[46]. An Analysis of Molecular Variance (AMOVA) to determine population differentiation based on genetic distance was carried out using the adonis2 function from the VEGAN package in R for all variables[47] and country was looked at using an amova with the POPPR package as this gave details of the variance explained within and between samples and populations[45], from which a *P* value was calculated using a randomization test with 999 permutations. The genetic distance computed for the amova was also used for Principal Components Analysis (PCA) using the *prcomp* function in R. To identify population clusters, a Discriminant Analysis of Principle Components (DAPC) was carried out after clusters were identified de novo (i.e., no prior location information) using the *find.clusters* function in the ADEGENET package in R[26]. Optimum number of K was selected based on BIC (Fig. 3A). The number of PCs retained in the DAPC was 7, this was determined using the a-score with the *optim.a.score* function in the ADEGENET package in R (Supplementary

Fig. S4 online). STRUCTURE (v. 2.3.4) was also used to identify population clusters, using an admixture model with 100,000 burnin and 100,000 reps for K1 to K15 with 15 iterations per K[48]. STRUCTURE results were visualised using STRUCTURE HARVESTER[49], CLUMPP[50] and DISTRUCT[51].

Received: 9 July 2021; Accepted: 7 October 2021
Published online: 21 October 2021

References

1. CABI. *Fall Armyworm (FAW) Portal*. www.cabi.org/isc/fallarmyworm (2020).
2. Westbrook, J., Nagoshi, R., Meagher, R., Fleischer, S. & Jairam, S. Modeling seasonal migration of fall armyworm moths. *Int. J. Biometeorol.* **60**, 255–267. https://doi.org/10.1007/s00484-015-1022-x (2016).
3. Nagoshi, R. & Meagher, R. Review of fall armyworm (Lepidoptera: noctuidae) genetic complexity and migration. *Fla. Entomol.* **91**, 546–554. https://doi.org/10.1653/0015-4040-91.4.546 (2008).
4. Nagoshi, R. N., Meagher, R. L. & Jenkins, D. A. Puerto Rico fall armyworm has only limited interactions with those from Brazil or Texas but could have substantial exchanges with Florida populations. *J. Econ. Entomol.* **103**, 360–367. https://doi.org/10.1603/EC09253 (2010).
5. Johnson, S. J. Migration and the life history strategy of the fall armyworm, *Spodoptera frugiperda* in the western hemisphere. *Int. J. Trop. Insect Sci.* **8**, 543–549. https://doi.org/10.1017/S1742758400022591 (1987).
6. Abrahams, P. *et al. Fall Armyworm: Impacts and Implications for Africa. Evidence Note 2* (CABI, 2017).
7. Nagoshi, R. N. *et al.* Fall armyworm migration across the Lesser Antilles and the potential for genetic exchanges between North and South American populations. *PLoS ONE* **12**, e0171743. https://doi.org/10.1371/journal.pone.0171743 (2017).
8. Arias, O. *et al.* Population genetic structure and demographic history of *Spodoptera frugiperda* (Lepidoptera: Noctuidae): Implications for insect resistance management programs. *Pest Manag. Sci.* **75**, 2948–2957. https://doi.org/10.1002/ps.5407 (2019).
9. Nagoshi, R. *et al.* Analysis of strain distribution, migratory potential, and invasion history of fall armyworm populations in northern Sub-Saharan Africa. *Sci. Rep.* **8**, 3710–3710. https://doi.org/10.1038/s41598-018-21954-1 (2018).
10. Nagoshi, R. N., Adamczyk, J. J., Meagher, R. L., Gore, J. & Jackson, R. Using stable isotope analysis to examine fall armyworm (Lepidoptera: Noctuidae) host strains in a cotton habitat. *J. Econ. Entomol.* **100**, 1569. https://doi.org/10.1603/0022-0493(2007)100[1569:USIATE]2.0.CO2 (2007).
11. Nagoshi, R. N. *et al.* Southeastern Asia fall armyworms are closely related to populations in Africa and India, consistent with common origin and recent migration. *Sci. Rep.* **10**, 1421. https://doi.org/10.1038/s41598-020-58249-3 (2020).
12. Nagoshi, R. N. *et al.* Genetic characterization of fall armyworm infesting South Africa and India indicate recent introduction from a common source population. *PLoS ONE* **14**, e0217755. https://doi.org/10.1371/journal.pone.0217755 (2019).
13. Nayyar, N. *et al.* Population structure and genetic diversity of invasive Fall Armyworm after 2 years of introduction in India. *Sci. Rep.* **11**, 7760. https://doi.org/10.1038/s41598-021-87414-5 (2021).
14. Zhang, L. *et al.* Genetic structure and insecticide resistance characteristics of fall armyworm populations invading China. *Mol. Ecol. Resour.* **20**, 1682–1696. https://doi.org/10.1111/1755-0998.13219 (2020).
15. Raymond, L., Plantegenest, M. & Vialatte, A. Migration and dispersal may drive to high genetic variation and significant genetic mixing: The case of two agriculturally important, continental hoverflies (*E. pisyrphus balteatus* and *S. phaerophoria scripta*). *Mol. Ecol.* **22**, 5329–5339. https://doi.org/10.1111/mec.12483 (2013).
16. Stevens, L. *et al.* Migration and gene flow among domestic populations of the Chagas insect vector *Triatoma dimidiata* (Hemiptera: Reduviidae) detected by microsatellite loci. *J. Med. Entomol.* **52**, 419–428. https://doi.org/10.1093/jme/tjv002 (2015).
17. Arias, R. S., Blanco, C. A., Portilla, M., Snodgrass, G. L. & Scheffler, B. E. First microsatellites from *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and their potential use for population genetics. *Ann. Entomol. Soc. Am.* **104**, 576–587. https://doi.org/10.1603/an10135 (2011).
18. Pavinato, V. A., Martinelli, S., de Lima, P. F., Zucchi, M. I. & Omoto, C. Microsatellite markers for genetic studies of the fall armyworm, *Spodoptera frugiperda*. *Genet. Mol. Res.: GMR* https://doi.org/10.4238/2013.February.8.1 (2013).
19. Nagoshi, R., Silvie, P. & Meagher, R. Comparison of haplotype frequencies differentiate fall armyworm (Lepidoptera: Noctuidae) corn-strain populations from Florida and Brazil. *J. Econ. Entomol.* **100**, 954–961 (2007).
20. Agapow, P.-M. & Burt, A. Indices of multilocus linkage disequilibrium. *Mol. Ecol. Notes* **1**, 101–102. https://doi.org/10.1046/j.1471-8278.2000.00014.x (2001).
21. Weir, B. S. *Genetic Data Analysis II: Methods for Discrete Population Genetic Data* (Sinauer, 1996).
22. Nei, M. Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci.* **70**, 3321. https://doi.org/10.1073/pnas.70.12.3321 (1973).
23. Hedrick, P. W. A standardized genetic differentiation measure. *Evolution* **59**, 1633–1638. https://doi.org/10.1111/j.0014-3820.2005.tb01814.x (2005).
24. Jost, L. O. U. GST and its relatives do not measure differentiation. *Mol. Ecol.* **17**, 4015–4026. https://doi.org/10.1111/j.1365-294X.2008.03887.x (2008).
25. Evanno, G., Regnaut, S. & Goudet, J. Detecting the number of clusters of individuals using the software structure: A simulation study. *Mol. Ecol.* **14**, 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x (2005).
26. Jombart, T. adegenet: A R package for the multivariate analysis of genetic markers. *Bioinformatics* **24**, 1403–1405. https://doi.org/10.1093/bioinformatics/btn129 (2008).
27. Nagoshi, R. N. *et al.* Comparative molecular analyses of invasive fall armyworm in Togo reveal strong similarities to populations from the eastern United States and the Greater Antilles. *PLoS ONE* **12**, e0181982. https://doi.org/10.1371/journal.pone.0181982 (2017).
28. Nagoshi, R. N., Goergen, G., Plessis, H. D., van den Berg, J. & Meagher, R. Genetic comparisons of fall armyworm populations from 11 countries spanning sub-Saharan Africa provide insights into strain composition and migratory behaviors. *Sci. Rep.* **9**, 8311. https://doi.org/10.1038/s41598-019-44744-9 (2019).
29. Buès, R., Bouvier, J. C. & Boudinhon, L. Insecticide resistance and mechanisms of resistance to selected strains of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in the south of France. *Crop Prot.* **24**, 814–820. https://doi.org/10.1016/j.cropro.2005.01.006 (2005).
30. Armes, N. J., Jadhav, D. R. & DeSouza, K. R. A survey of insecticide resistance in Helicoverpa armigera in the Indian subcontinent. *Bull. Entomol. Res.* **86**, 499–514. https://doi.org/10.1017/S0007485300039298 (1996).
31. Parry, H. R. *et al.* Estimating the landscape distribution of eggs by Helicoverpa spp., with implications for Bt resistance management. *Ecol. Model.* **365**, 129–140. https://doi.org/10.1016/j.ecolmodel.2017.10.004 (2017).
32. Jones, C. M., Parry, H., Tay, W. T., Reynolds, D. R. & Chapman, J. W. Movement ecology of pest Helicoverpa: Implications for ongoing spread. *Annu. Rev. Entomol.* **64**, 277–295. https://doi.org/10.1146/annurev-ento-011118-111959 (2019).

33. Tucker, M. R., Mwandoto, S. & Pedgley, D. E. Further evidence for windborne movement of armyworm moths, *Spodoptera exempta*, in East Africa. *Ecol. Entomol.* **7**, 463–473. https://doi.org/10.1111/j.1365-2311.1982.tb00689.x (1982).
34. Rose, D. J. W. *et al.* Downwind migration of the African army worm moth, *Spodoptera exempta*, studied by mark-and-capture and by radar. *Ecol. Entomol.* **10**, 299–313. https://doi.org/10.1111/j.1365-2311.1985.tb00727.x (1985).
35. Rose, D. J. W., Dewhurst, C. F. & Page, W. W. *The African Armyworm Handbook: The Status, Biology, Ecology, Epidemiology and Management of Spodoptera exempta (Lepidoptera: Noctuidae)* (Natural Resources Institute, 2000).
36. Chapman, J. W., Reynolds, D. R. & Wilson, K. Long-range seasonal migration in insects: Mechanisms, evolutionary drivers and ecological consequences. *Ecol. Lett.* **18**, 287–302. https://doi.org/10.1111/ele.12407 (2015).
37. Nagoshi, R. N. & Meagher, R. L. Using intron sequence comparisons in the triose-phosphate isomerase gene to study the divergence of the fall armyworm host strains. *Insect Mol. Biol.* **25**, 324–337. https://doi.org/10.1111/imb.12223 (2016).
38. Hall, T. A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acid Symp. Ser.* **41**, 95–98 (1999).
39. Thompson, J. D., Higgins, D. G. & Gibson, T. J. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* **22**, 4673–4680. https://doi.org/10.1093/nar/22.22.4673 (1994).
40. R Core Team. *R Foundation for Statistical Computing* (R Core Team, 2020).
41. Paradis, E. pegas: An R package for population genetics with an integrated–modular approach. *Bioinformatics* **26**, 419–420. https://doi.org/10.1093/bioinformatics/btp696 (2010).
42. Adamack, A. & Gruber, B. PopGenReport: Simplifying basic population genetic analyses in R. *Methods Ecol. Evol.* https://doi.org/10.1111/2041-210X.12158 (2014).
43. Goudet, J. Hierfstat, a package for r to compute and test hierarchical F-statistics. *Mol. Ecol. Notes* **5**, 184–186. https://doi.org/10.1111/j.1471-8286.2004.00828.x (2005).
44. Winter, D. MMOD: An R library for the calculation of population differentiation statistics. *Mol. Ecol. Resour.* https://doi.org/10.1111/j.1755-0998.2012.03174.x (2012).
45. Kamvar, Z. N., Tabima, J. F. & Grünwald, N. J. Poppr: An R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* **2**, e281. https://doi.org/10.7717/peerj.281 (2014).
46. Raymond, M. & Rousset, F. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *J. Hered.* **86**, 248–249. https://doi.org/10.1093/oxfordjournals.jhered.a111573 (1995).
47. Oksanen, J. *et al.* Vegan: Community Ecology Package. R package version 2.0-2. (2012).
48. Pritchard, J. K., Stephens, M. & Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959 (2000).
49. Earl, D. A. & von Holdt, B. M. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **4**, 359–361. https://doi.org/10.1007/s12686-011-9548-7 (2012).
50. Jakobsson, M. & Rosenberg, N. A. CLUMPP: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**, 1801–1806. https://doi.org/10.1093/bioinformatics/btm233 (2007).
51. Rosenberg, N. A. DISTRUCT: A program for the graphical display of population structure. *Mol. Ecol. Notes* **4**, 137–138. https://doi.org/10.1046/j.1471-8286.2003.00566.x (2004).

Acknowledgements

We would like to thank all those who helped collect fall armyworm samples in Africa, including Donald Kachigamba, Aislinn Pearson, Sevgan Subramanian, Kentosse Gutu Ouma, Catherine Adongo Awuoche, Patrick Karangwa, Bellancile Uzayisenga, Benjamin Mensah and Samuel Adjei Mensah. Natural Environment Research Council Envision DTP Grant (NE/L002604/1) awarded to KW, CMJ and AW. Global Challenges Research Fund Grant (BB/P023444/1) awarded to KW.

Author contributions

A.J.W. drafted the manuscript, all authors read and commented on the manuscript. A.J.W., C.M.J., J.A.S., K.W. were involved in the conception and design of the paper. All authors were involved in the acquisition of data. A.J.W. analysed and interpreted the data with support from C.M.J., J.A.S. and K.W.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-00298-3.

Correspondence and requests for materials should be addressed to A.J.W. or K.W.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 6 March 2020 | Revised: 23 June 2020 | Accepted: 25 June 2020

DOI: 10.1111/1755-0998.13219

RESOURCE ARTICLE

MOLECULAR ECOLOGY RESOURCES WILEY

Genetic structure and insecticide resistance characteristics of fall armyworm populations invading China

Lei Zhang[1] | Bo Liu[1] | Weigang Zheng[1] | Conghui Liu[1] | Dandan Zhang[2] | Shengyuan Zhao[2] | Zaiyuan Li[1] | Pengjun Xu[3,4] | Kenneth Wilson[4,1] | Amy Withers[4] | Christopher M. Jones[5] | Judith A. Smith[6] | Gilson Chipabika[7] | Donald L. Kachigamba[8] | Kiwoong Nam[9] | Emmanuelle d'Alençon[9] | Bei Liu[1] | Xinyue Liang[1] | Minghui Jin[1] | Chao Wu[1] | Swapan Chakrabarty[1] | Xianming Yang[2] | Yuying Jiang[10] | Jie Liu[10] | Xiaolin Liu[11] | Weipeng Quan[12] | Guirong Wang[1] | Wei Fan[1] | Wanqiang Qian[1] | Kongming Wu[2] | Yutao Xiao[1]

[1]Shenzhen Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Genome Analysis Laboratory of the Ministry of Agriculture, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, China

[2]State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China

[3]Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, China

[4]Lancaster Environment Centre, Lancaster University, Lancaster, UK

[5]Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Blantyre, Malawi

[6]School of Forensic and Applied Sciences, University of Central Lancashire, Preston, UK

[7]Zambia Agriculture Research Institute (ZARI), Lusaka, Zambia

[8]Department of Agricultural Research Services (DARS), Bvumbwe Research Station, Limbe, Malawi

[9]DGIMI, Univ. Montpellier, INRA, Montpellier, France

[10]National Agro-Tech Extension and Service Center, Beijing, China

[11]Novogene Bioinformatics Institute, Beijing, China

[12]Grandomics Biosciences, Co., Ltd, Beijing, China

Correspondence
Yutao Xiao, Shenzhen Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Genome Analysis Laboratory of the Ministry of Agriculture, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, China.
Email: xiaoyutao@caas.cn

Kongming Wu, State Key Laboratory for Biology of Plant Diseases and Insect PestsInstitute of Plant ProtectionChinese, Academy of Agricultural Sciences, Beijing,China.
Email: wukongming@caas.cn

Wanqiang Qian, Shenzhen Branch, Guangdong Laboratory for Lingnan Modern

Abstract

The rapid wide-scale spread of fall armyworm (*Spodoptera frugiperda*) has caused serious crop losses globally. However, differences in the genetic background of subpopulations and the mechanisms of rapid adaptation behind the invasion are still not well understood. Here we report the assembly of a 390.38-Mb chromosome-level genome of fall armyworm derived from south-central Africa using Pacific Bioscience (PacBio) and Hi-C sequencing technologies, with scaffold N50 of 12.9 Mb and containing 22,260 annotated protein-coding genes. Genome-wide resequencing of 103 samples and strain identification were conducted to reveal the genetic background of fall armyworm populations in China. Analysis of genes related to pesticide- and *Bacillus thuringiensis* (Bt) resistance showed that the risk of fall armyworm developing

Lei Zhang, Bo Liu, Weigang Zheng, Conghui Liu, Dandan Zhang and Shengyuan Zhao are contributed equally to this work.

Agriculture, Genome Analysis Laboratory of the Ministry of Agriculture, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, China.
Email: qianwanqiang@caas.cn

Wei Fan, Shenzhen Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Genome Analysis Laboratory of the Ministry of Agriculture, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, China.
Email: fanwei@caas.cn

Funding information
Key Project for Breeding Genetic Modified Organisms, Grant/Award Number: 2016ZX08012004-003 and 2019ZX08012004-002; Shenzhen Science and Technology Program, Grant/Award Number: KQTD20180411143628272; UK's Global Challenges Research Fund and Biotechnology and Biological Sciences Research Council, Grant/Award Number: BB/P023444/1; UK Natural Environment Research Council Envision Doctoral Training Programme, Grant/Award Number: NE/L002604/1; Key-Area Research and Development Program of Guangdong Province, Grant/Award Number: 2020B020223004

resistance to conventional pesticides is very high. Laboratory bioassay results showed that insects invading China carry resistance to organophosphate and pyrethroid pesticides, but are sensitive to genetically modified maize expressing the Bt toxin Cry1Ab in field experiments. Additionally, two mitochondrial fragments were found to be inserted into the nuclear genome, with the insertion event occurring after the differentiation of the two strains. This study represents a valuable advance toward improving management strategies for fall armyworm.

KEYWORDS
gene insertion, population structure, resistance risk, *Spodoptera frugiperda*, subpopulations

1 | INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a polyphagous pest that is native to tropical and subtropical America, with a strong capacity for migration and reproduction (Johnson, 1987; Mitchell et al., 1991; Westbrook, Nagoshi, Meagher, Fleischer, & Jairam, 2016). It was first detected in Africa in 2016 (Goergen, Kumar, Sankung, Togola, & Tamò, 2016) and spread to 44 African countries within 2 years. It was detected in India in 2018, and has now spread to several southeastern Asian countries (Nagoshi et al., 2020). Such rapid spread poses a global threat to food production. The strong environmental adaptability of fall armyworm is not only reflected in its polyphagy for a wide range of host plants (Luginbill, 1928), but also in its evolution of resistance to chemical pesticides and genetically modified crops expressing *Bacillus thuringiensis* (Bt) toxins (Bernardi et al., 2015; Leibee & Capinera, 1995; Monnerat et al., 2015; Signorini et al., 2018; Storer et al., 2010). Studies have shown that gene families related to detoxification and metabolic processes in the fall armyworm have clearly expanded (Gouin et al., 2017; Liu et al., 2019). In addition, there are two morphologically identical, but genetically distinct, subpopulations or strains of fall armyworm, the rice-strain (R-strain) and the corn-strain (C-strain), which differ in their host plant selection and sex pheromone composition (Lima & McNeil, 2009; Pashley, 1986; Pashley, Hammond, & Hardy, 1992; Pashley & Martin, 1987). However, there is no absolute mating barrier between the two strains and productive hybridization has been confirmed in both laboratory and field studies (Dumas et al., 2015; Nagoshi, Meagher, Nuessly, & Hall, 2006).

To date, several field-evolved resistant populations of fall armyworm have been detected, including those displaying resistance to a variety of chemical pesticides and Bt crops (Chandrasena et al., 2018; Gutiérrez-Moreno et al., 2019; Zhu et al., 2015). The reported mechanisms of resistance to pesticides are mainly due to variation in receptor genes, such as amino acid changes in the ryanodine receptor (RyR) (diamide), acetylcholinesterase (AChE) (organophosphate) and voltage-gated sodium channel (VGSC) (pyrethroids) (Boaventura et al., 2020; Carvalho, Omoto, Field, Williamson, & Bass, 2013; Yu, Nguyen, & Abo-Elghar, 2003). In addition, the frameshift mutation resulting in early termination of the ATP-dependent Binding Cassette subfamily C2 gene (*ABCC2*) gene, caused by a 2-bp insertion, is linked to resistance to Bt toxin Cry1Fa (Banerjee et al., 2017). Field-evolved strains resistant to Bt toxin Vip3Aa20 were obtained by screening homozygous resistance loci in F_2 generations in the laboratory (Yang et al., 2018). Clarifying the development of pesticide- and Bt-resistance in fall armyworm would be helpful in providing scientific support for the commercialization of genetically modified crops and Bt biopesticides.

Recent studies have indicated that molecular identification of the C- and R-strains of fall armyworm is dependent on which markers are used (Meagher & Gallo-Meagher, 2003; Nagoshi, 2012). Early molecular markers based on mitochondrial *Cytochrome Oxidase Subunit I* (*COI*) and Z-chromosome-linked *Tpi* genes failed to

accurately assign the strain genetic background (Juárez et al., 2014; Nagoshi, 2019; Nagoshi, Goergen, Goergen, Du Plessis, van den Berg, & Meagher, 2019; Nagoshi et al., 2017). The dominant populations of fall armyworm invading Africa and Asia were speculated to be hybrid populations based on these two molecular markers (Zhang et al., 2019). In addition, an Africa-specific haplotype, different from those native to the Americas, was also reported in African and Chinese samples based on the *Tpi* gene (Liu et al., 2019; Nagoshi et al., 2019), which makes strain identification and studies of population genetic structure more complicated. Therefore, a genome-wide analysis of the genetic characteristics of invasive fall armyworm is becoming imperative. Although several versions of the fall armyworm genome have now been published (Gouin et al., 2017; Kakumani, Malhotra, Mukherjee, & Bhatnagar, 2014; Liu et al., 2019; Nam et al., 2019; Nandakumar, Ma, & Khan, 2017), a high-quality genome assembly from a different geographical source is a valuable addition to the genomic resources for this species. Moreover, the different biological properties of the C- and R-strains and the debate regarding strain identification will benefit from further genomic support and explanation. Here we report a chromosome-level genome sequence of a male moth from an inbred fall armyworm strain, which derived from field populations collected in Zambia in 2017 and would be classed as C-strain based on *COI* genotype but possessed an Africa-specific *Tpi* haplotype which differs from the Western Hemisphere (henceforth American) R- and C-strain. We also resequenced 103 fall armyworm samples from 16 Provinces in China, as well as four samples collected from two African countries (Zambia and Malawi). The genome-wide genetic backgrounds of the invading fall armyworm samples were compared, and insecticide-resistance risk was assessed based on analysis of potential resistance-related genes. Comparative genomic analyses of these data will help to reveal the resistance-related mechanisms and the population genetic characteristics of fall armyworm, which may facilitate its future management.

2 | MATERIALS AND METHODS

2.1 | Samples and sequencing for genome assembly

The fall armyworm samples were collected from maize fields in Lusaka, Zambia, in 2017 and reared to produce an inbred strain. One male moth, derived from seven successive generations of single-pair sib mating, was selected for genomic sequencing for the primary assembly data set and all other individuals used in the Hi-C and RNAseq experiments were from the same inbred strain. DNA was extracted using the Qiagen Genomic DNA kit (Cat. no. 13323, Qiagen) followed by purity assessment and quantification with a NanoDrop One UV-Vis spectrophotometer (Thermo Fisher Scientific) and Qubit 3.0 Fluorometer (Invitrogen), respectively. About 0.5 μg genomic DNA (gDNA) was used as input to generate a PCR-free Illumina genomic library using the Truseq Nano DNA HT Sample preparation Kit (Illumina), with 350-bp insert size and this library was sequenced in 2 × 150-bp format on the Illumina NovaSeq 6000 platform. Five micrograms of gDNA from the same individual was used as an input for ~20-kb insert libraries (SMRTbell Template Prep Kit 1.0, Cat. no. 100-259-100, PacBio) sequenced on the PacBio Sequel (Pacific Biosciences). Two third-instar larvae were selected for Hi-C library construction, and nuclear DNA was cross-linked in situ, extracted and then digested with the restriction enzyme *Dpn*II. Hi-C libraries were amplified by 12–14 cycles of PCR and sequenced on the Illumina NovaSeq 6000 platform with 2 × 150-bp reads. In addition, three fifth-instar larvae, three pupae, three female moths and three male moths were used for RNA sequencing. Total RNA was extracted using the RNeasy Mini extraction kit (Qiagen), and a NanoPhotometer spectrophotometer (Implen) and Qubit 2.0 Flurometer (Life Technologies) were used to check the purity and concentration of RNA, respectively. One microgram total RNA per sample was used to make indexed cDNA libraries using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB) following the manufacturer's recommendations. The libraries had insert sizes of 250–300 bp and were sequenced on the Illumina NovaSeq 6000 platform with 150-bp paired-end output.

2.2 | Genome assembly and correction

The raw PacBio reads longer than 5 kb were assembled into contigs using the software WTDBG2 version 2.4 with the parameters "-p 0 -k 15 -AS 2 -s 0.05 -L 5000" (Ruan & Li, 2019). ARROW version 2.1.0 (https://github.com/PacificBiosciences/GenomicConsensus) was used to correct assembly errors after comparing contigs with PacBio reads using PBALIGN version 0.4.1 (https://github.com/PacificBiosciences/pbalign). The Illumina raw reads were filtered by trimming the adapter and low-quality regions using CLEAN _ ADAPTER version 1.1 with the parameter "-a Both-adapter -r 75 -s 12" and CLEAN _ LOWQUAL version 1.0 with the parameter "-e 0.001 -r 75" (https://github.com/fanagislab/ assembly_2ndGeneration/tree/master/clean_illumina). The filtered Illumina reads were aligned to the assembled contigs by BWA MEM version 0.7.17 (Li & Durbin, 2009), and single base errors in the contigs were corrected by PILON version 1.21 (Walker et al., 2014).

2.3 | Genome estimation and evaluation

A distribution analysis of 17 k-mer frequencies was performed to estimate the genome size of fall armyworm. The filtered Illumina reads were used as input to construct k-mer frequencies by JELLYFISH (https://github.com/gmarcais/Jellyfish). Genome size was estimated using G = K_num/K_depth, where the K_num is the total number of K-mers, and K_depth is the frequency occurring more frequently than the others (Li et al., 2010). We used the arthropoda gene set (odb9) to assess the integrity of the genome by Benchmarking Universal Single-Copy Ortholog (BUSCO) version 3.0.2 (Simao, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015).

2.4 | Chromsome assembly based on Hi-C data

The Hi-C sequencing raw reads were filtered to remove reads containing <5 bases of adaptor sequence; >50% of bases with phred quality value of <19; and <5% of unknown bases (N). Filtered reads were then aligned to the assembled contigs using BOWTIE2 (version 2.2.3; http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) (Langmead & Salzberg, 2012). Invalid read pairs were filtered using default settings by HIC-PRO (version 2.7.8; https://github.com/nservant/HiC-Pro) (Servant et al., 2015). LACHESIS (https://github.com/shendurelab/LACHESIS) (Burton et al., 2013) was applied to cluster, order and orient contigs based on the agglomerative hierarchical clustering algorithm. For each chromosome cluster, the ordered contigs were oriented by building a weighted, directed acyclic graph (WDAG). The orientation of each contig in each chromosomal group was predicted according to the maximum-likelihood path through WDAG. Finally, we cut the chromosomes predicted by LACHESIS into bins of equal length (100 kb) and constructed a heatmap based on the interaction signals revealed by valid mapped read pairs between bins using HIC-PRO.

2.5 | Gene prediction and annotation

A de novo repeat library of fall armyworm was constructed by REPEATMODELER version 1.0.4 (http://www.repeatmasker.org/RepeatModeler.html). Transposable elements (TEs) were identified by REPEATMASKER version 4.0.6 (http://www.repeatmasker.org/) using both the de novo library and Repbase library (Repbase-20150923), and tandem repeats were predicted using TANDEM REPEATS FINDER (Benson, 1999) version 4.07b. We used a combination of ab initio prediction, homology searches and RNA-seq annotation to predict genes in the *Spodeptera frugiperda* genome. We performed ab initio prediction using AUGUSTUS 2.5.5 with default parameters (Stanke & Waack, 2003). For homology-based annotation, we queried the *S. frugiperda* genome sequences against a database containing nonoverlapping protein sequences from closely related species (*Bombyx mori*, *Helicoverpa armigera*, *Spodoptera litura*) by GENBLASTA with default parameters (She, Chu, Wang, Pei, & Chen, 2009). GENEWISE (Birney, Clamp, & Durbin, 2004) was used to refine the GENBLASTA mappings to the genome. For the RNA-seq annotation, the RNA-seq data were mapped to the assembled genome of *S. frugiperda* using TOPHAT version 2.0.12 and alignments were processed by CUFFLINKS version 2.2.1 with default parameters to generate transcript predictions (Trapnell et al., 2012). EVIDENCE MODELER (Haas et al., 2008) version 1.1.1 was used to combine ab initio predictions, homology-based searches and RNA-seq alignments. Predicted gene models supported by at least one of the annotations using the UniProt database, NR database and RNA-seq data were retained. Gene functional annotation was performed by aligning the predicted protein sequences to the NCBI NR, UniProt, eggNOG, and KEGG databases with BLASTP version 2.3.0+, applying an *E*-value cut-off < 10^{-5}.

2.6 | Phylogenetic tree construction and genomic comparison

Orthologous and paralogous gene families identified in a set of 10 species (*Drosophila melanogaster*, *Plutella xylostella*, *Bombyx mori*, *Manduca sexta*, *Danaus plexippus*, *Heliconius melpomene*, *Operophtera brumata*, *Helicoverpa armigera*, *Spodoptera frugiperda*, *Spodoptera litura*) with published genomes were analysed by ORTHOFINDER version 2.3.1 with default parameters. Orthologous groups that contain single-copy genes for each species were selected to construct the phylogenetic tree. The multisequence alignment of proteins was accomplished by MUSCLE (Edgar, 2004) version 3.8.31. A neighbour-joining (NJ) phylogenetic tree was constructed using MEGA version 7.0.14. The current assembled genome was aligned with two published versions of fall armyworm genomes using the MUMMER3.23 (Kurtz et al., 2004) package with cutoff of identity >80% and coverage >80%. Alignments were filtered to generate a multi-alignment data set using the delta-filter utility with 85% minimum identity (-i 85) and minimum alignment length 10 (-l 10). A set of unique alignments was created using the same filter criteria but with the addition of the -r and -q flags.

2.7 | Sampling for resequencing and population genetic study

A total of 103 Chinese fall armyworm samples were used for resequencing. All samples were collected as larvae on maize or sugarcane from 50 cities of 16 provinces (autonomous regions or municipalities) of China. The larvae were fed with fresh maize leaves and brought back to the laboratory under ambient conditions during transportation. Larval bodies were cleaned and then stored in a freezer at −80°C. Detailed sample information is presented in Table S1 and the sample distribution in China is shown in Figure S1. In addition, four fall armyworm samples from Africa were also used for resequencing, including two samples (AFR4 and 5) from the same inbred strain (AFR2017) as the genome sequencing in this study, and another two samples (AFR14 and 15) which were collected from maize fields in Bvumbwe, Malawi, in January 2019, which is also an inbred strain (AFR2019) reared in the laboratory. A total of 1.5 μg gDNA of each sample was used to construct a 350-bp insert library using the Truseq Nano DNA HT Sample preparation Kit (Illumina) sequenced in 150-bp paired-end mode as described in section 2.1. Raw reads were aligned to the NCBI NT database using BLASTN, and reads with significant matches (identity > 95% and coverage > 80%) to microbes or host plants were removed.

A further 173 fall armyworm samples from 21 provinces in China were used for strain identification and molecular detection using PCR amplification and Sanger sequencing. Genomic DNA was extracted using the Multisource Genomic DNA Miniprep Kit (Axygen) according to product instructions. The 50-μl PCR mixture contained 25 μl of 2 × Easytaq mix, a mixture of 2 μl forward and reverse primers (10 μM), 2 μl DNA, and 21 μl diethy1 pyrocarbonate (DEPC) H_2O.

PCR was performed at 94°C for 5 min, 34 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and finally 72°C for 5 min. A total of 10 μl of the PCR products containing the target fragment were sequenced by Life Technology. These samples were collected from the field as larvae or adult moths. Detailed sample information is presented in Table S2 and the sample distribution in China is shown in Figure S1. Mitochondrial *COI* and *Tpi* markers were used for strain identification. ABCC2 and AChE genes were detected based on primers designed according to published mutation sites (Banerjee et al., 2017; Carvalho et al., 2013). Inserted mitochondrial fragments in the nuclear genome were detected using primers designed in this study. All primer sequence information in this study is shown in Table S3.

2.8 | Read mapping and SNP calling

The Illumina raw reads from resequenced samples were filtered using CLEAN _ ADAPTER and CLEAN _ LOWQUAL software as described in section 2.1, resulting in high-quality reads with an average error rate of <0.01. The high-quality reads were then aligned to the fall armyworm reference genome (American C-strain) and mitochondrial genome sequences using BWA MEM software (Li & Durbin, 2009) version 0.7.5a with default parameters. Alignments for each sample were processed by removing duplicate reads using the SAMTOOLS (Li et al., 2009) software package version 1.3. The mpileup function in SAMTOOLS was used to generate mpileup files for each sample. VCFTOOLS (Li, 2011) was used to identify SNPs and small indels. Several criteria were considered in SNP filtering: (a) a read mapping score higher than 40; (b) minimum coverage greater than 10; and (c) SNP genotypes called in >90% of samples. We also conducted principal component analysis (PCA) to evaluate genetic structure using the software Genome-wide Complex Trait Analysis (GCTA) version 1.04 (Yang, Lee, Goddard, & Visscher, 2011).

2.9 | Bioassays of insecticides and Bt maize in the field

Bioassays were conducted by a topical application procedure (Armes, Jadhav, Bond, & King, 1992). Two inbred strains (cdcc and cdyc) collected from Yunnan Province and reared for multiple generations in the laboratory were tested using 14 types of pesticide commonly used in agricultural production (Table S4). Drops (1.0 μl) of a serial dilution of technical insecticides in acetone solution were applied with a micropipette to the thoracic dorsum of the third-instar larvae, with control larvae treated with 1.0 μl acetone. After treatment, the larvae were reared individually in 24-well plates containing ad libitum artificial diet without any Bt proteins or insecticides. Larvae were retained in an insect chamber with a controlled environment of 26 ± 1°C, 60 ± 10% relative humidity and a photoperiod of 16 hr: 8 hr (light–dark). Mortality was assessed after 72 hr of treatment. Larvae were considered dead if they were unable to move in a coordinated manner when prodded with a small soft brush. We used median lethal doses (LC_{50}) to evaluate the resistance level of different fall armyworm populations. The LC_{50} and 95% fiducial limit (FL) for each insecticide were estimated by probit analysis using the software package POLO-PC (Russell, Robertson, & Savin, 1977) (LeOra Software).

The Bt toxin field bioassays were conducted at a genetically modified (GM) test base in Yunnan Province, China. Test seeds of GM maize (expressing Cry1Ab) and control maize were provided by the DBN Biotech Center, Beijing DBN Technology Group Co., Ltd. Both maize types were planted in ~180 m^2, with each type being replicated three times. Larval density and maize damage rates were investigated at different growth stages of maize at seven different dates during June and July. The investigation was performed in a five-spot sampling method with 20 maize plants per point. Fall armyworm damage assessment was performed according to standard procedures (Davis, Ng, & Williams, 1992; Williams, Buckley, & Daves, 2006; Wiseman & Widstrom, 1984).

3 | RESULTS

3.1 | High-quality genome assembly of fall armyworm

A total of 25.89 Gb raw PacBio long reads and 162.4 Gb Illumina raw reads were generated. After filtering low-quality and duplicated reads, 24.72 Gb PacBio long reads and 95.4 Gb high-quality Illumina reads were used for genome assembly, together representing an ~300× coverage of the fall armyworm genome. Using WTDBG2 (Ruan & Li, 2019), the final genome was assembled into 776 contigs with size of 390.38 Mb and contig N50 length of 5.6 Mb (longest, 18.5 Mb), including a complete mitochondrial sequence (Table 1). The assembled genome size was close to the estimated size of 395 Mb based on k-mer depth distribution analysis, which was also similar to that determined by flow cytometry (396 ± 3 Mb) (Gouin et al., 2017). After interaction analysis based on a total of 78 Gb data obtained through Hi-C sequencing, 143 contigs were concatenated into 31 linkage groups with a scaffold N50 of 12.9 Mb, accounting for 96.3% of the total genome length (Figure 1). By aligning the Illumina data with the assembled fall armyworm genome, the mapping rate and coverage were 98.8% and 99.7% (≥5 reads) respectively, highlighting the accuracy and high integrity of the genome assembly. The genome size reported in this study is intermediate between those of previously published fall armyworm versions, but the genome is nearly 140 Mb smaller than that recently published by Liu et al. (2019). Genome collinearity analysis showed that more than 98% of the current assembled genome was consistent with previously published versions (Gouin et al., 2017; Liu et al., 2019) (Table S5), and regions within the assembly presented in this study align to multiple regions of Liu's assembly, indicating the previous assembled genome with larger size was mainly caused by high heterozygosity of sequenced samples.

TABLE 1 Summary of assembly results of *Spodoptera frugiperda*

Assembly feature	FAW (this study)	FAW (corn strain)	FAW (rice strain)
Assembled sequences (Mb)	390	438	371
Longest scaffold size (kb)	21,916.7	943.2	314.1
N50 size of scaffold (kb)	12,966.7	52.8	28.5
N90 size of scaffold (kb)	7,574.2	3.5	6.4
Longest contig size (kb)	18,555.4	362.9	191.4
N50 size of contig (kb)	5,606.9	16.9	24.3
N90 size of contig (kb)	991.8	2.9	5.6
GC content in genome (%)	36.4	36.0	36.1
Number of gene models	22,260	21,700	26,329
BUSCO complete gene (%)	98.4	88.1	93.5
BUSCO duplicated gene (%)	2	11.3	2
BUSCO missing gene (%)	1.4	4.2	2.3

By combining homology-based and de novo approaches, we identified ~27.2% of repetitive elements in the assembled fall armyworm genome. Among the known repeat families, long interspersed nuclear elements (LINE) constituted the most abundant repeat family, representing 8.7% of the repetitive sequences, while long terminal repeats (LTR) comprised only 1.4% (Table S6). To annotate the fall armyworm genome, we performed deep transcriptome sequencing of larvae, pupae, and male and female moths, including three different developmental stages, which generated 98.4 Gb of RNA-seq data. By combining homologue-based, ab initio and transcriptome-based approaches, we predicted 22,260 protein-coding genes (gene models) in the fall armyworm genome, which is greater than the number of predicted genes in other lepidopteran genomes that have so far been published (Dasmahapatra et al., 2012; Kanost et al., 2016; Pearce et al., 2017; Wan et al., 2019; Xia et al., 2008; You et al., 2013; Zhan, Merlin, Boore, & Reppert, 2011). More than 85.5% of the predicted coding sequences (CDS) were supported by transcriptome sequencing data (defined as when ≥ 70% of the predicted CDS of a gene was covered by transcriptome reads). Further assessment of assembly integrity based on BUSCO analysis showed that the current genome contained 98.4% complete BUSCO genes.

Comparative analysis of orthogroups of nine Lepidoptera species and *Drosophila melanogaster* (Diptera) was performed (Table S7). Among them, 17,180 genes in 10,755 orthogroups were found in the current genome of fall armyworm, and the remaining 5,080 lineage-specific genes were identified as unassigned genes. Compared with *Spodoptera litura*, *S. frugiperda* has more species-specific genes, and the number of unassigned genes is much greater than that of *S. litura* (Figure 2a). Phylogenomic analyses of the 10 species were conducted using 1,571 single-copy genes. As shown in Figure 2a, the taxonomic relationship and phylogenetic status of current species was similar to phylogenetic analyses based on 13 mitochondrial protein-coding genes (Lämmermann, Vogel, & Traut, 2016). Three species of Noctuidae, including *S. frugiperda*, formed one group, which then clustered with *Bombyx mori* (Bombycidae) and *Manduca sexta* (Sphingidae). Two butterflies, *Danaus plexippus* and *Heliconius melpomene* (both Nymphalidae), clustered together as an outer branch, while *Plutella xylostella* (Plutellidae) is the outermost branch of Lepidoptera (Figure 2a).

3.2 | Genetic background of fall armyworm populations in China

A total of 103 fall armyworm samples from China were resequenced, as well as four samples from two countries in Africa (Zambia and Malawi). The generated Illumina data ranged from 8.6 to 18.9 Gb for each sample, with a median genome coverage of 32.5×. First, we analysed the whole mitochondrial genome sequences of all samples. A total of 208 SNP loci were selected for analysis, based on comparison of the published mitochondrial sequences of both the American R-strain (AXE) and C-strain (ASW) (Gouin et al., 2017). Genotypes were obtained at these 208 sites for each individual after mapping the filtered sequence reads to the assembled mitochondrial genome. We found that most of the samples were assigned to the R-strain, and all four samples from Africa were C-strain, while only four out of 103 samples in China were assigned to the C-strain based on the mitochondrial genome (Figure 3a). Note that most R-strain samples surprisingly contain heterozygous mitochondrial SNPs, which could be caused by inserted C-strain fragments or existing standing variation of low frequency. The proportion of the C-strain in this sample set was ~10% and was similar to that of the 173 Chinese fall armyworm samples identified by PCR based on the *COI* gene in this study (Table S2).

Next, we analysed the *Tpi* gene, which is commonly used in strain identification of fall armyworm (Nagoshi, 2012). By comparing the full-length *Tpi* gene of the American R-strain (AXE) and C-strain (ASW), 22 SNP loci were found. The genotype of each individual was analysed based on these 22 sites. The results showed that all fall armyworm samples collected from China contained more C-strain SNP loci, as did the Malawi samples (AFR14, AFR15), but not those from Zambia samples (AFR4, AFR5) which represents the Africa-specific haplotype and which contained ~50% of R-strain SNP loci. Genotypes of seven Chinese samples were identical to the American C-strain (ASW) and the remaining samples contained a small proportion of R-strain genotypes or heterozygous SNPs (Figure 3b). However, none of the samples was found to be identical to the American R-strain genotype (AXE). We further used PCR to analyse genotypes of 173 samples based on 10 strain-biased SNPs within the *Tpi* gene reported previously (Nagoshi, 2012). The results showed that almost all of the samples correspond to C-strain genotypes, although three samples (G-GXW11, G-GXW13, G-EP6) were identified as an Africa-specific haplotype, which was significantly different from known R- or C-strain genotypes (Figure 4; Table S2). In summary, our genotyping results show that there are obvious contradictions between strain identification using mitochondrial and *Tpi* gene markers.

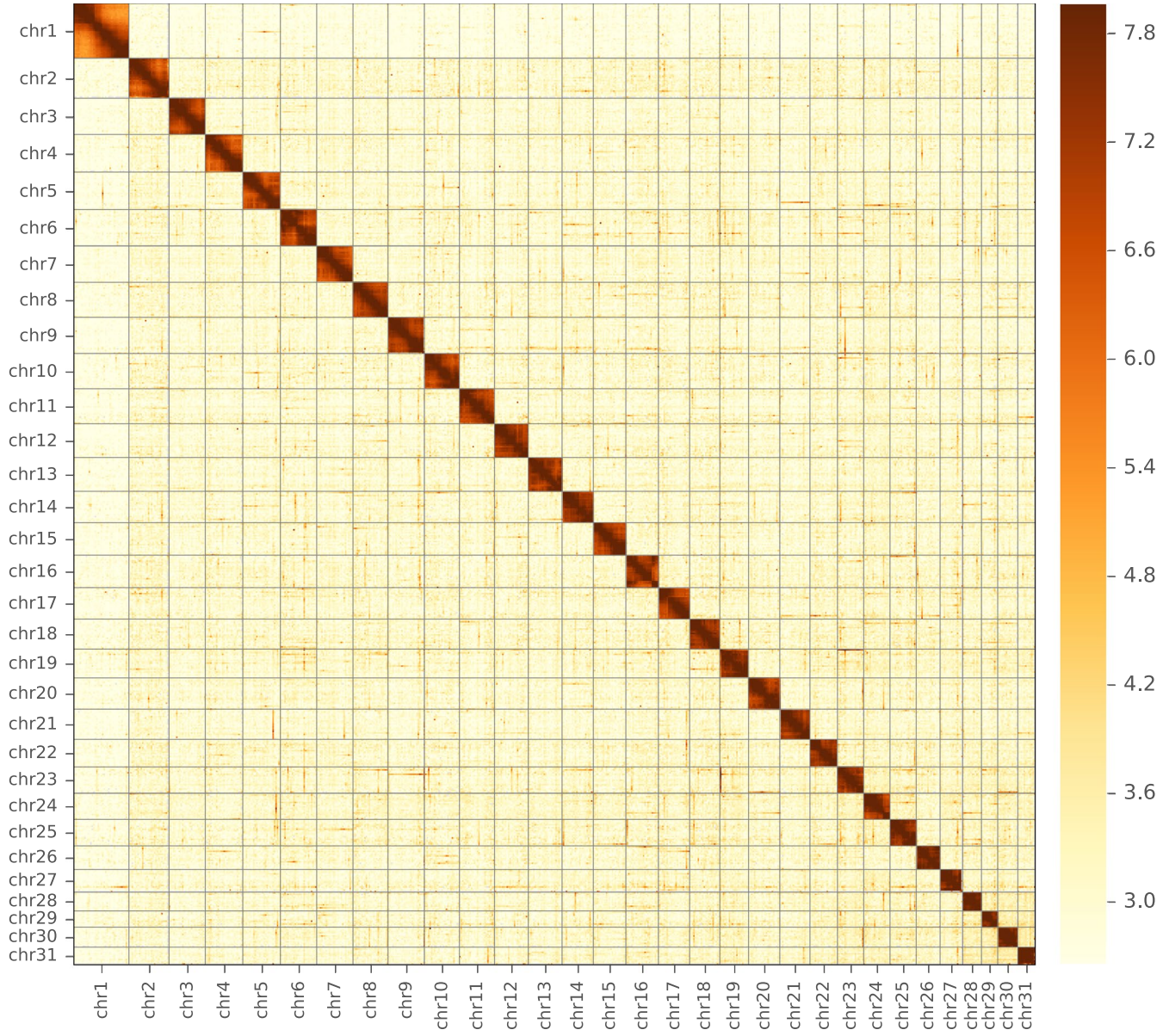


FIGURE 1 A genome-wide contact matrix from Hi-C data between each pair of the 31 chromosomes [Colour figure can be viewed at wileyonlinelibrary.com]

To clarify the genetic background of fall armyworm populations invading China, we screened a total of 707,353 SNPs exhibiting homozygous differences between the reference American R-strain (AXE) and C-strain (ASW) in the 107 resequenced samples (Figure 3c). The results showed that all the samples, including the four from Africa, had more than 70% of the genetic background of the American C-strain (ASW) genotype. The proportion of R-strain SNPs was about 15%, and the remaining 12% were heterozygous. The results showed that fall armyworm invading China have a dominant percentage of the C-strain background. PCA based on 5,998,089 whole-genome SNPs also demonstrated that samples from China were much closer to the C-strain (ASW) than to the R-strain (AXE), in which PC1 explained 6.45% of the variation. African samples from Zambia (AFR4, AFR5) were separated on PC2, which explained 2.15% of the variation (Figure 3d). By comparing the results of the mitochondrial genome, *Tpi* gene and genome-wide identification, it becomes apparent that there is no correlation between the mitochondrial and whole genome genotype. Although *Tpi* genotyping shows results more similar to those of the whole genome, the presence of the Africa-specific *Tpi* haplotype increases the complexity of using this marker for identification.

3.3 | Fall armyworm is developing a high risk of resistance to conventional pesticides

Insecticide resistance evolution is one of the most challenging problems in the control of fall armyworm. Identifying resistance-related genes is helpful for the monitoring and prevention of fall armyworm outbreaks. We selected 14 previously reported resistance-related genes of lepidopteran pests and scanned the resequenced samples to analyse variation in

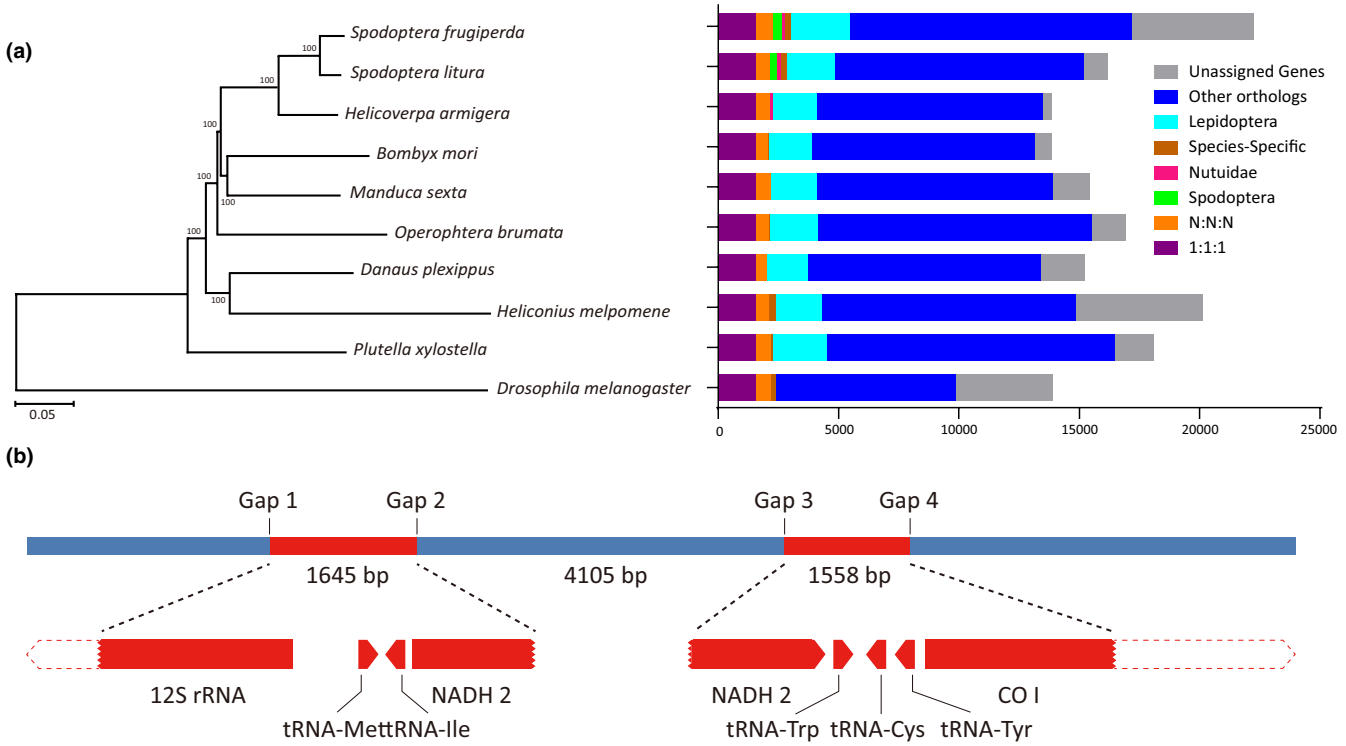


FIGURE 2 Phylogenetic relationships and schematic map of mitochondrial insertion. (a) Phylogenetic tree and genomic comparison of 10 species of Lepidoptera and Diptera. *Drosophila melanogaster* was used as an outgroup and bootstrap value was set as 1,000, 1:1:1 includes the common orthologues with the same number of copies in different species, N:N:N includes the common orthologues with different copy numbers in different species, other orthologues include the unclassified orthologues, and unassigned genes include the genes that cannot be clustered into known gene families. (b) A schematic map of two mitochondrial fragments inserted into the nuclear genome; the *NADH2* gene was separated by a 4,105-bp fragment, and both of two inserted mitochondrial fragments were identical with the C-strain genotype [Colour figure can be viewed at wileyonlinelibrary.com]

target genes. The results showed that all the target genes had multiple variation sites with a high frequency of SNPs in the CDS region (Table S8).

Studies have shown that the amino acid substitutions in AChE (A201S, G227A, F290V), VGSC (T929I, L932F, L1014F) and RyR (I4790M, G4946E) result in resistance to organophosphate, pyrethroid and diamide insecticides, respectively. The results of variation scanning of the 107 resequenced samples showed that resistance mutations were present in amino acids 201 and 290 of AChE (Figure 5a). Among them, the first locus had 17.1% heterozygous mutations, and the third locus had 29.7% homozygous resistance mutations and 58.2% heterozygous mutations. No resistance mutations were detected at the targeted sites of the VGSC and RyR gene in any samples. We also designed primers to detect the resistance mutation sites in *AChE* in 173 Chinese samples by PCR amplification and Sanger sequencing. The results were similar to the Illumina data, showing that ~75% of samples have homozygous or heterozygous variation at amino acid 290.

To understand the baseline susceptibility of fall armyworm invading China, we determined the LC_{50} values to 14 insecticides for two Chinese fall armyworm populations collected from Yunnan Province. The results showed that the LC_{50} for both fall armyworm populations to chlorpyrifos, a fenvalerate, were at relatively high level, and well above those of the laboratory-susceptible *Helicoverpa armigera* strain (Bird & Downes, 2014). The LC_{50} to chlorantraniliprole was low, as were those to emamectin benzoate and indoxacarb, which were similar to results of a previous study on *H. armigera* and could be considered as the susceptible baseline (Bird, 2015) (Figure 6). Resistance levels of the two populations to pyrethroids and organophosphate pesticides were very high; in particular, resistance ratios to chlorpyrifos of the two populations were more than 300-fold compared to a laboratory susceptible fall armyworm population that was sampled in 1975 (Yu, 1991) (Figure 5b). These results provide a susceptible baseline for fall armyworm populations invading China to different pesticides, which can provide guidance for resistance monitoring and pesticide management strategies.

3.4 | Fall armyworm invading China are currently sensitive to Bt toxin in a field-evolved experiment

The insertion of 2 bp in the *ABCC2* gene of fall armyworm was reported to cause a frame-shift mutation and results in resistance to Cry1Fa (Banerjee et al., 2017). We did not detect the same insertion mutation in 107 resequenced samples nor in 173 samples screened by using PCR and Sanger sequencing. Although the percentage of SNPs in the CDS region of other Bt receptors such as *SR-C* (scavenger receptor class C gene, a specific receptor for Vip3Aa in Sf9 cells), *TSPAN1* and other ABC gene families related to Cry toxin were also

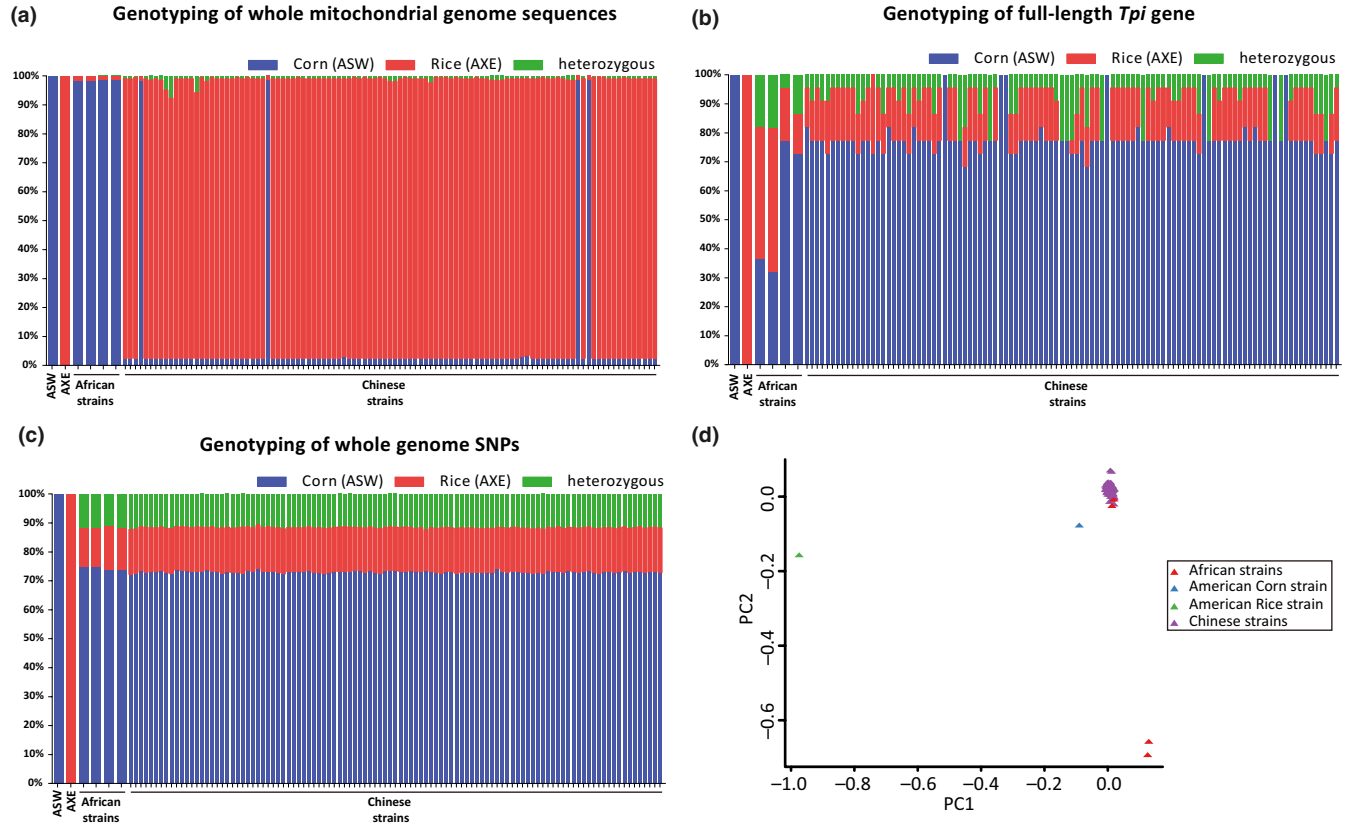


FIGURE 3 Genetic background of 107 fall armyworm samples. (a) Genotyping based on 208 mitochondrial SNP loci. From left to right, the leftmost two samples are ASW (the American corn strain) and AXE (the American rice strain), four African strains (AFR4 and 5 from Zambia, then AFR14 and 15 from Malawi), and 103 strains from China; the order of each sample is consistent with Table S1. (b) Genotyping based on 22 SNP loci in the *Tpi* gene. (c) Genotyping based on 707,353 genome SNP loci. (d) Principal component analysis (PCA) based on 5,998,089 whole-genome SNPs. Colour codes indicate samples from different sources; the two samples at the bottom in red are African samples AFR4 and AFR5 [Colour figure can be viewed at wileyonlinelibrary.com]

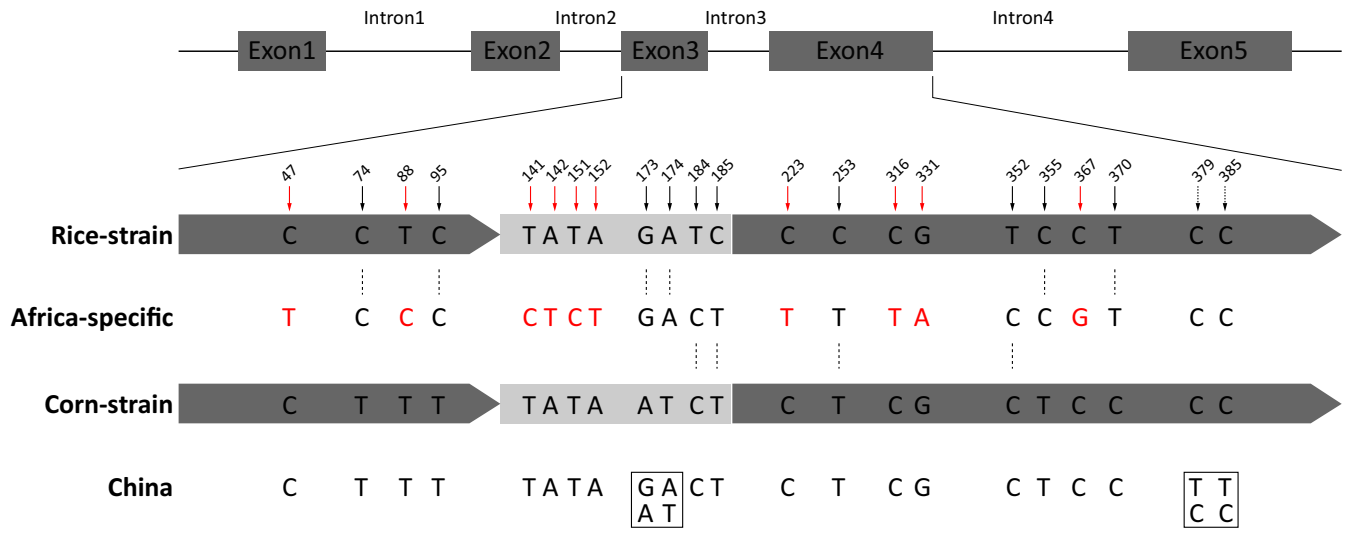


FIGURE 4 Diagram of the *Tpi* gene segments with respect to consensus Western Hemisphere sequences and the haplotypes observed in samples collected from Africa and China. Black solid arrows indicate 10 SNPs used to identify the American R-strain and C-strain fall armyworm, in which P370 was considered to be an effective diagnostic marker. Red solid arrows indicate 10 SNPs specific to the Africa-specific strain. The boxes represent two variable loci in some Chinese samples, including homozygous or heterozygous genotypes [Colour figure can be viewed at wileyonlinelibrary.com]

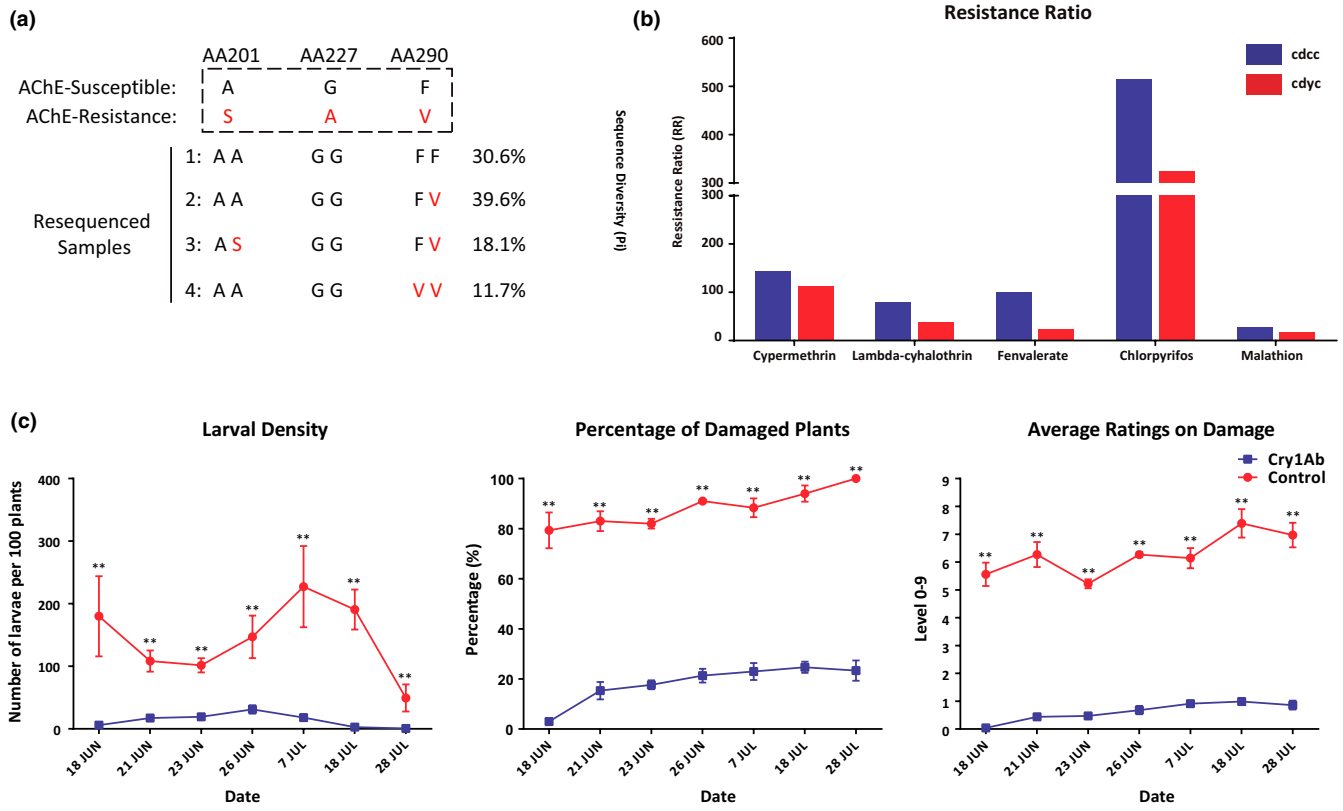


FIGURE 5 Genome scans and bioassays of fall armyworm for insecticide resistance. (a) Genotype and resistance mutation sites of the AChE gene in fall armyworm populations in China. (b) The resistance ratios (RRs) of two Chinese fall armyworm populations to pyrethroid (cypermethrin, lambda-cyhalothrin, fenvalerate) and organophosphate (chlorpyrifos, malathion) insecticides; cdcc and cdyc represent two inbred strains collected from Yunnan Province in China. RRs were calculated from the LD_{50} (μg/g) of a field population over the LD_{50} of a susceptible population as in Yu (1991). (c) Resistance tests of GM maize and non-GM maize to fall armyworm in field experiments. Error bars are the *SD* ($n = 15$), and asterisks indicate significant differences based on Student's *t* test (**$p < .01$) [Colour figure can be viewed at wileyonlinelibrary.com]

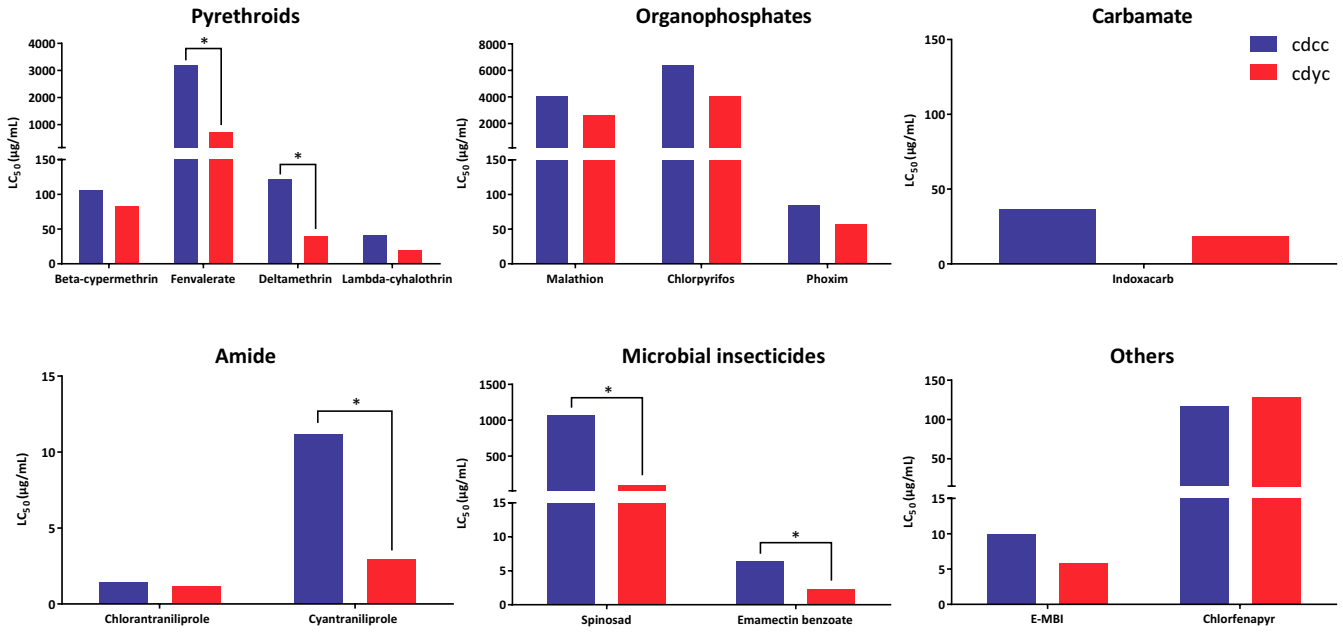


FIGURE 6 The LC_{50} values of two Chinese fall armyworm populations to different kinds of insecticides. cdcc and cdyc represent two inbred strains collected from Yunnan Province in China. Statistical significance of the difference was assessed by whether the 95% fiducial limits overlap (*$p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

very high (Table S8), no reported resistant mutation was found in any target resistance genes.

Field tests showed that fall armyworm samples invading China were sensitive to GM maize expressing Cry1Ab compared with the control group. Damage assessment on larval density, the percentage of damaged plants and average damage ratings of GM maize were significantly lower than those of the control group (Figure 5c), indicating that the GM maize expressing Cry1Ab currently has good control effects on the invading population of fall armyworm in China.

3.5 | Insertion of mitochondrial fragments into the nuclear genome in a recent evolution event

We found that two mitochondrial fragments, with sequence lengths of 1.5 kb (partial *COI* gene and *NADH2* gene) and 1.6 kb (partial *NADH2* gene and 12S rRNA gene), were inserted into the nuclear genome, separated by a 4.1-kb segment of the nuclear genome (Figure 2b). The total length of a ~ 7.3-kb fragment, including two inserted fragments, was supported by more than 28 raw reads of PacBio data. The lengths of all 28 reads were longer than 20 kb and completely covered the 7.3-kb fragment. However, the two insertions were not found in other published fall armyworm genomes. To verify the accuracy of this result, we designed four primers based on flanking sequences of four connection points (Gap1–4 in Figure 2b), and the results of PCR amplification confirmed the existence of the insertion. The same primers were used in PCR assays to detect the insertion in 173 fall armyworm samples and it was found that the insertion was present in only 26.0% of all samples (Table S2). At the same time, the resequencing data of 107 fall armyworm samples in this study also showed that there were varying numbers of reads covering the four junction points in 29 samples, and the percentage of samples with inserted reads was 27.1% (Table S9). Both the PCR and resequencing results showed that the insertion was not present in all samples, perhaps suggesting that it has a recent evolutionary origin.

Moreover, the genotype of the two inserted mitochondrial fragments was identical to that of the C-strain, indicating that the insertion occurred after differentiation of the R- and C-strains. Further analysis indicated that the two mitochondrial fragments were inserted into the intron region of the lysine-specific demethylase 3 B (*Kdm3B*) gene, which is not likely to affect expression of the gene. The inserted partial *COI* and *NADH2* gene fragments were also considered likely to be functionless.

4 | DISCUSSION

The rapid spread of the fall armyworm has attracted popular attention worldwide. Accurate identification of its genetic characteristics (strain and pesticide resistance properties) has a direct and practical importance in terms of risk assessment and control strategies. A genome-wide analysis can reveal more in-depth genetic information than conventional gene-level analyses. The results of this study show that the fall armyworm invading China has a genetic background dominated by American corn-strain genotypes. Most of the fall armyworm samples invading China were detected and collected from corn and sugarcane, which are more likely to show the characteristics of C-strain host plants. Along the invasion path of the migratory fall armyworm, there are large-scale rice planting areas in Southeast Asia and central China, although there are few reports of serious damage to rice caused by fall armyworm (http://www.fao.org/fall-armyworm). The established R-strain fall armyworm in the Americas mainly feeds on turf grass, and there were few reports of damage to rice in 1970s (Bowling, 1978; Gallego, 1967). In addition, the established R-strain *Tpi* genotype has not been detected in any of the samples collected from Africa or Asia. We therefore speculate that the American R-strain fall armyworm did not invade Africa or Asia, including China.

In our study, 103 resequenced Chinese samples were collected from different regions of 50 cities distributed across 16 provinces (Figure S1). The collection time and sites coincided almost perfectly with the spreading invasion of fall armyworm in China. However, there was no obvious correlation between the time or site of collection and the genetic structure of the fall armyworm population (Figure 3). Almost all samples have similar genomic backgrounds, which suggests that the invading population may originate from a single genetic source and there is no evidence for genomic selection during the invasion.

According to our results, commonly used strain identification of fall armyworms by mitochondrial or *Tpi* markers is limited or even inaccurate. The nuclear insertion of two C-strain partial *COI* fragments in this study further underlines the need for caution in interpreting mitochondrial genotypes. We also found that the AT/GC SNP located at *Tpi*-intron3 (P173/174) was inadequate as a diagnostic marker. In addition, the TT/CC SNP located at *Tpi*-exon4 (P379/385) was associated with sequence variation in *Tpi*-intron4 (Figure 4; Figure S2), which could further be developed as a marker to subdivide C-strain samples. It is noteworthy that a particular (Africa-specific) haplotype of the *Tpi* gene originally identified in Africa was tentatively designated as R-strain based on the $E4^{183}$ site (equal to P370 in Figure 4 in this study) in previous studies (Nagoshi, 2012). Our genome-wide SNP analysis revealed that this haplotype contained more C-strain SNPs than R-strain SNPs.

The sample used for the genome sequencing in this study represents a combination of the particular *Tpi* haplotype and C-strain *COI*. We also found combinations of the R-strain *COI* and particular *Tpi* (sample G-XW13), as well as heterozygous forms of the particular *Tpi* and *Tpi*-C with the R-strain *COI* in two samples (G-GXW11, G-EP6). These combinations of different genotypes show that the genetic boundaries between two established (American) R- and C-strains are obscure. The insertion of two mitochondrial fragments into the nuclear genome might be caused by random hybridization between different genotypes, which would suggest that fall armyworm invading China might be descendants of an interstrain hybrid population. This is the first report of DNA fragments transferred from mitochondria into the nuclear genome in a *Spodoptera* lineage, and two such fragments

could be used to develop markers to identify specific populations and to follow further evolutionary events of fall armyworm.

The rapid evolution of insecticide resistance and the increasing levels of resistance observed in fall armyworm populations needs attention. In this study, reported mutations related to insecticide resistance were detected in the AChE gene. Although some mutation sites were detected as heterozygous in most samples, the frequency of resistant mutation sites will increase greatly under the selection pressure caused by application of related pesticides in the field. The bioassay results showed that armyworms invading China have evolved high levels of resistance to organophosphate pesticides, which was consistent with the results of molecular scanning of resistance-related genes, yet the resistance to pyrethroid pesticides cannot be explained by any reported mechanism. However, the fall armyworms invading China are currently sensitive to GM maize expressing Cry1Ab in field experiments, and are also sensitive to other Bt toxins in the laboratory, according to previous studies (Li et al., 2019). At present, GM maize shows better application prospects in controlling fall armyworm in China, as larval density and damage rate of GM maize were significantly less than that of non-GM plants, although this crop is currently not registered for use in China.

This study provides a high-quality reference genome that demonstrates a genomic feature different from the established (American) C- or R-strain genotypes, as well as more comprehensive gene annotation. We also present resequencing data for 103 fall armyworm individuals invading China. The samples cover different regions and times during 2019, providing basic materials for analysing global population genetic and identifying patterns of invasiveness. Baseline resistance data for Chinese fall armyworm populations are shown to 14 common pesticides, providing guidance for the control and resistance monitoring of fall armyworm. Small-scale field experiments in this study suggest that fall armyworm in China are currently susceptible to GM maize, and these results could provide an important application reference for commercial planting of Bt maize in China. There are other important issues that remain for further exploitation using this whole genome approach, such as identifying the genes involved in polyphagy, migratory capability and olfaction, which could provide valuable tools for the future management of fall armyworms.

ACKNOWLEDGEMENTS

The following bodies provided funding that contributed to this work: Key Project for Breeding Genetic Modified Organisms (grants 2016ZX08012004-003, 2019ZX08012004-002), Shenzhen Science and Technology Program (KQTD20180411143628272), the UK's Global Challenges Research Fund and Biotechnology and Biological Sciences Research Council (BB/P023444/1), the UK Natural Environment Research Council Envision Doctoral Training Programme (NE/L002604/1), and Key-Area Research and Development Program of Guangdong Province (2020B020223004).

AUTHOR CONTRIBUTIONS

Y.X., K.W., W.Q. and W.F. conceived the project, designed content and managed the project; L.Z. and G.W. coordinated the project; B.L., Z.L. and X. Liu. performed read mapping, SNP calling and population analysis; W.Z. and C.L. performed genome assembly and annotation; L.Z. and B.L. performed Hi-C assembly; D.Z. performed the laboratory bioassay; S.Z. performed the field experiment; P.X. performed transcriptome analysis; K.N. and E.A. provided the raw data of American R- and C-strains; B. Liu., X.L., M.J., C.W. and X.Y. performed the DNA extraction, PCR and sequence variation analysis; W.Q. constructed DNA libraries, performed sequencing; Y.J. and J.L. collected and provided samples from China; L.Z. wrote the manuscript; K.W., A.W., C.M.J., J.A.S., G.C., D.L.K. and S.C. revised the manuscript. All authors commented on the manuscript.

DATA AVAILABILITY STATEMENT

This Whole Genome Shotgun project of *Spodoptera frugiperda* has been deposited at DDBJ/ENA/GenBank under accession no. WUTJ00000000 with BioProject ID PRJNA591441. The version described in this paper is version WUTJ01000000. Raw sequencing reads of PacBio, RNA-seq, Hi-C and resequencing in this paper can be accessed at ftp://ftp.agis.org.cn/Spodoptera_Frugiperda/.

ORCID

Lei Zhang https://orcid.org/0000-0003-0809-5836
Kenneth Wilson https://orcid.org/0000-0001-5264-6522
Christopher M. Jones https://orcid.org/0000-0002-6504-6224
Swapan Chakrabarty https://orcid.org/0000-0001-7096-5690
Wei Fan https://orcid.org/0000-0001-5036-8733

REFERENCES

Armes, N. J., Jadhav, D. R., Bond, G. S., & King, A. B. (1992). Insecticide resistance in *Helicoverpa armigera* in South India. *Pesticide Science*, *34*(4), 355–364.

Banerjee, R., Hasler, J., Meagher, R., Nagoshi, R., Hietala, L., Huang, F., … Jurat-Fuentes, J. L. (2017). Mechanism and DNA-based detection of field-evolved resistance to transgenic Bt corn in fall armyworm (*Spodoptera frugiperda*). *Scientific Reports*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-09866-y

Benson, G. (1999). Tandem repeats finder: A program to analyze DNA sequences. *Nucleic Acids Research*, *27*(2), 573–580. https://doi.org/10.1093/nar/27.2.573

Bernardi, O., Bernardi, D., Ribeiro, R. S., Okuma, D. M., Salmeron, E., Fatoretto, J., … Omoto, C. (2015). Frequency of resistance to Vip3Aa20 toxin from Bacillus thuringiensis in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. *Crop Protection*, *76*, 7–14. https://doi.org/10.1016/j.cropro.2015.06.006

Bird, L. J. (2015). Baseline Susceptibility of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to Indoxacarb, Emamectin Benzoate, and Chlorantraniliprole in Australia. *Journal of Economic Entomology*, *108*(1), 294–300. https://doi.org/10.1093/jee/tou042

Bird, L. J., & Downes, S. (2014). Toxicity and Cross-Resistance of Insecticides to Cry2Ab-Resistant and Cry2Ab-Susceptible *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology*, *107*(5), 1923–1930.

Birney, E., Clamp, M., & Durbin, R. (2004). GeneWise and genomewise. *Genome Research*, *14*, 988–995. https://doi.org/10.1101/gr.1865504

Boaventura, D., Bolzan, A., Padovez, F. E., Okuma, D. M., Omoto, C., & Nauen, R. (2020). Detection of a ryanodine receptor target-site mutation in diamide insecticide resistant fall armyworm, *Spodoptera frugiperda*. *Pest Management Science*, *76*(1), 47–54.

Bowling, C. C. (1978). Simulated insect damage to rice: Effects of leaf removal. *Journal of Economic Entomology*, *71*(2), 377–378. https://doi.org/10.1093/jee/71.2.377

Burton, J. N., Adey, A., Patwardhan, R. P., Qiu, R., Kitzman, J. O., & Shendure, J. (2013). Chromosome-scale scaffolding of de novo genome assemblies based on chromatin interactions. *Nature Biotechnology*, *31*(12), 1119–1127. https://doi.org/10.1038/nbt.2727

Carvalho, R. A., Omoto, C., Field, L. M., Williamson, M. S., & Bass, C. (2013). Investigating the molecular mechanisms of organophosphate and pyrethroid resistance in the fall armyworm *Spodoptera frugiperda*. *PLoS One*, *8*(4), e62268. https://doi.org/10.1371/journal.pone.0062268

Chandrasena, D. I., Signorini, A. M., Abratti, G., Storer, N. P., Olaciregui, M. L., Alves, A. P., & Pilcher, C. D. (2018). Characterization of field-evolved resistance to Bacillus thuringiensis-derived Cry1F δ- endotoxin in *Spodoptera frugiperda* populations from Argentina. *Pest Management Science*, *74*(3), 746–754.

Dasmahapatra, K. K., Walters, J. R., Briscoe, A. D., Davey, J. W., Whibley, A., Nadeau, N. J., … Salazar, C. (2012). Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. *Nature*, *487*(7405), 94–98.

Davis, F. M., Ng, S. S., & Williams, W. P. (1992). Visual rating scales for screening whorl-stage corn for resistance to fall armyworm. *Technical bulletin-Mississippi Agricultural and Forestry. Experiment Station(USA)*, *186*, 1–9.

Dumas, P., Legeai, F., Lemaitre, C., Scaon, E., Orsucci, M., Labadie, K., … Aury, J. M. (2015). *Spodoptera frugiperda* (Lepidoptera: Noctuidae) host-plant variants: Two host strains or two distinct species? *Genetica*, *143*(3), 305–316. https://doi.org/10.1007/s10709-015-9829-2

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Gallego, F. L. (1967). Lista preliminar de insectos de importancia económica y secundarios, que afectan los principales cultivos, animales domésticos y al hombre, en colombia. *Revista Facultad Nacional De Agronomía Medellín*, *26*(65), 32–66.

Goergen, G., Kumar, P. L., Sankung, S. B., Togola, A., & Tamò, M. (2016). First report of outbreaks of the fall armyworm *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera, Noctuidae), a new alien invasive pest in West and Central Africa. *PLoS One*, *11*(10), e0165632.

Gouin, A., Bretaudeau, A., Nam, K., Gimenez, S., Aury, J. M., Duvic, B., … Kuwar, S. (2017). Two genomes of highly polyphagous lepidopteran pests (*Spodoptera frugiperda*, Noctuidae) with different host-plant ranges. *Scientific Reports*, *7*(1), 1–12. https://doi.org/10.1038/s41598-017-10461-4

Gutiérrez-Moreno, R., Mota-Sanchez, D., Blanco, C. A., Whalon, M. E., Terán-Santofimio, H., Rodriguez-Maciel, J. C., & DiFonzo, C. (2019). Field-evolved resistance of the fall armyworm (Lepidoptera: Noctuidae) to synthetic insecticides in Puerto Rico and Mexico. *Journal of Economic Entomology*, *112*(2), 792–802. https://doi.org/10.1093/jee/toy372

Haas, B. J., Salzberg, S. L., Zhu, W., Pertea, M., Allen, J. E., Orvis, J., … Wortman, J. R. (2008). Automated eukaryotic gene structure annotation using EVidenceModeler and the Program to Assemble Spliced Alignments. *Genome Biology*, *9*(1), 1–22. https://doi.org/10.1186/gb-2008-9-1-r7

Johnson, S. J. (1987). Migration and the life history strategy of the fall armyworm, *Spodoptera frugiperda* in the Western Hemisphere. *International Journal of Tropical Insect Science*, *8*(4-5-6), 543–549. https://doi.org/10.1017/S1742758400022591

Juárez, M. L., Schöfl, G., Vera, M. T., Vilardi, J. C., Murúa, M. G., Willink, E., … Groot, A. T. (2014). Population structure of *Spodoptera frugiperda* maize and rice host forms in South America: Are they host strains? *Entomologia Experimentalis Et Applicata*, *152*(3), 182–199.

Kakumani, P. K., Malhotra, P., Mukherjee, S. K., & Bhatnagar, R. K. (2014). A draft genome assembly of the army worm, *Spodoptera frugiperda*. *Genomics*, *104*(2), 134–143. https://doi.org/10.1016/j.ygeno.2014.06.005

Kanost, M. R., Arrese, E. L., Cao, X., Chen, Y. R., Chellapilla, S., Goldsmith, M. R., … Papanicolaou, A. (2016). Multifaceted biological insights from a draft genome sequence of the tobacco hornworm moth, *Manduca sexta*. *Insect Biochemistry and Molecular Biology*, *76*, 118–147. https://doi.org/10.1016/j.ibmb.2016.07.005

Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C., & Salzberg, S. L. (2004). Versatile and open software for comparing large genomes. *Genome Biology*, *5*(2), 1–9.

Lämmermann, K., Vogel, H., & Traut, W. (2016). The mitochondrial genome of the Mediterranean flour moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae), and identification of invading mitochondrial sequences (numts) in the W chromosome. *European Journal of Entomology*, *113*, 482–488. https://doi.org/10.14411/eje.2016.063

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Leibee, G. L., & Capinera, J. L. (1995). Pesticide resistance in Florida insects limits management options. *Florida Entomologist*, *78*, 386–399. https://doi.org/10.2307/3495525

Li, G. P., Ji, T., Sun, X., Jiang, Y., Wu, K., & Feng, H. Q. (2019). Susceptibility evaluation of invaded *Spodoptera frugiperda* population in Yunnan province to five Bt proteins. *Plant Protection*, *45*(3), 15–20.

Li, H. (2011). A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics*, *27*(21), 2987–2993. https://doi.org/10.1093/bioinformatics/btr509

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, *25*(14), 1754–1760. https://doi.org/10.1093/bioinformatics/btp324

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … Durbin, R. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Li, R., Fan, W., Tian, G., Zhu, H., He, L., Cai, J., … Wang, J. (2010). The sequence and de novo assembly of the giant panda genome. *Nature*, *463*(7279), 311–317.

Lima, E. R., & McNeil, J. N. (2009). Female sex pheromones in the host races and hybrids of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Chemoecology*, *19*(1), 29–36. https://doi.org/10.1007/s00049-009-0005-y

Liu, H., Lan, T., Fang, D., Gui, F., Wang, H., Guo, W., …Sahu, S. K. (2019). Chromosome level draft genomes of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), an alien invasive pest in China. *BioRxiv*, 671560. http://dx.doi.org/10.1101/671560

Luginbill, P. (1928). The fall armyworm. U.S. Dept. Agric. Tech. Bull, *34*, 1–91.

Meagher, R. L. Jr, & Gallo-Meagher, M. (2003). Identifying host strains of fall armyworm (Lepidoptera: Noctuidae) in Florida using mitochondrial markers. *Florida Entomologist*, *86*(4), 450–455. https://doi.org/10.1653/0015-4040(2003)086[0450:IHSOFA]2.0.CO;2

Mitchell, E. R., McNeil, J. N., Westbrook, J. K., Silvain, J. F., Lalanne-Cassou, B., Chalfant, R. B., … Proshold, F. I. (1991). Seasonal periodicity of fall armyworm, (Lepidoptera: Noctuidae) in the Caribbean basin and northward to Canada. *Journal of Entomological Science*, *26*(1), 39–50. https://doi.org/10.18474/0749-8004-26.1.39

Monnerat, R., Martins, E., Macedo, C., Queiroz, P., Praca, L., Soares, C. M., … Bravo, A. (2015). Evidence of field-evolved resistance of *Spodoptera frugiperda* to Bt corn expressing Cry1F in Brazil that is still sensitive to modified Bt toxins. *PLoS One*, *10*(4), e0119544. https://doi.org/10.1371/journal.pone.0119544

Nagoshi, R. N. (2012). Improvements in the identification of strains facilitate population studies of fall armyworm subgroups. *Annals of the Entomological Society of America*, *105*(2), 351–358. https://doi.org/10.1603/AN11138

Nagoshi, R. N. (2019). Evidence that a major subpopulation of fall armyworm found in the Western Hemisphere is rare or absent in Africa, which may limit the range of crops at risk of infestation. *PLoS One*, *14*(4), e0208966. https://doi.org/10.1371/journal.pone.0208966

Nagoshi, R. N., Goergen, G., Du Plessis, H., van den Berg, J., & Meagher, R. (2019). Genetic comparisons of fall armyworm populations from 11 countries spanning sub-Saharan Africa provide insights into strain composition and migratory behaviors. *Scientific Reports*, *9*(1), 1–11. https://doi.org/10.1038/s41598-019-44744-9

Nagoshi, R. N., Htain, N. N., Boughton, D., Zhang, L., Xiao, Y., Nagoshi, B. Y., & Motasanchez, D. (2020). Southeastern Asia fall armyworms are closely related to populations in Africa and India, consistent with common origin and recent migration. *Scientific Reports*, *10*(1), 1–10. https://doi.org/10.1038/s41598-020-58249-3

Nagoshi, R. N., Koffi, D., Agboka, K., Tounou, K. A., Banerjee, R., Jurat-Fuentes, J. L., & Meagher, R. L. (2017). Comparative molecular analyses of invasive fall armyworm in Togo reveal strong similarities to populations from the eastern United States and the Greater Antilles. *PLoS One*, *12*(7), e0181982. https://doi.org/10.1371/journal.pone.0181982

Nagoshi, R. N., Meagher, R. L., Nuessly, G., & Hall, D. G. (2006). Effects of fall armyworm (Lepidoptera: Noctuidae) interstrain mating in wild populations. *Environmental Entomology*, *35*(2), 561–568. https://doi.org/10.1603/0046-225X-35.2.561

Nam, K., Gimenez, S., Hilliou, F., Blanco, C. A., Hänniger, S., Bretaudeau, A., …d'Alençon, E. (2019). Adaptation by copy number variation increases insecticide resistance in fall armyworms. *BioRxiv*. 812958. https://doi.org/10.1101/812958

Nandakumar, S., Ma, H., & Khan, A. S. (2017). Whole-genome sequence of the *Spodoptera frugiperda* Sf9 insect cell line. *Genome Announcements*, *5*(34), e00829–17. https://doi.org/10.1128/genomeA.00829-17

Pashley, D. P. (1986). Host-associated genetic differentiation in fall armyworm (Lepidoptera: Noctuidae): A sibling species complex? *Annals of the Entomological Society of America*, *79*(6), 898–904. https://doi.org/10.1093/aesa/79.6.898

Pashley, D. P., Hammond, A. M., & Hardy, T. N. (1992). Reproductive isolating mechanisms in fall armyworm host strains (Lepidoptera: Noctuidae). *Annals of the Entomological Society of America*, *85*(4), 400–405. https://doi.org/10.1093/aesa/85.4.400

Pashley, D. P., & Martin, J. A. (1987). Reproductive incompatibility between host strains of the fall armyworm (Lepidoptera: Noctuidae). *Annals of the Entomological Society of America*, *80*(6), 731–733. https://doi.org/10.1093/aesa/80.6.731

Pearce, S. L., Clarke, D. F., East, P. D., Elfekih, S., Gordon, K. H. J., Jermiin, L. S., … Rane, R. V. (2017). Genomic innovations, transcriptional plasticity and gene loss underlying the evolution and divergence of two highly polyphagous and invasive *Helicoverpa* pest species. *BMC Biology*, *15*(1), 63. https://doi.org/10.1186/s12915-017-0402-6

Ruan, J., & Li, H. (2019). Fast and accurate long-read assembly with wtdbg2. *Nature Methods*, *17*, 1–4. https://doi.org/10.1038/s41592-019-0669-3

Russell, R. M., Robertson, J. L., & Savin, N. E. (1977). POLO: A new computer program for probit analysis. *Bulletin of the ESA*, *23*(3), 209–213. https://doi.org/10.1093/besa/23.3.209

Servant, N., Varoquaux, N., Lajoie, B. R., Viara, E., Chen, C., Vert, J., … Barillot, E. (2015). HiC-Pro: An optimized and flexible pipeline for Hi-C data processing. *Genome Biology*, *16*(1), 259. https://doi.org/10.1186/s13059-015-0831-x

She, R., Chu, J. S. C., Wang, K., Pei, J., & Chen, N. S. (2009). genBlastA: Enabling BLAST to identify homologous gene sequences. *Genome Research*, *19*, 143–149. https://doi.org/10.1101/gr.082081.108

Signorini, A. M., Abratti, G., Grimi, D., Machado, M., Bunge, F. F., Parody, B., … Araujo, M. P. (2018). Management of field-evolved resistance to Bt maize in Argentina: A multi-institutional approach. *Frontiers in Bioengineering and Biotechnology*, *6*(67), 1–5. https://doi.org/10.3389/fbioe.2018.00067

Simao, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: Assessing genome assembly and annota- tion completeness with single-copy orthologs. *Bioinformatics*, *31*, 3210–3212. https://doi.org/10.1093/bioinformatics/btv351

Stanke, M., & Waack, S. (2003). Gene prediction with a hidden Markov model and a new intron submodel. *Bioinformatics*, *19*, 215–225. https://doi.org/10.1093/bioinformatics/btg1080

Storer, N. P., Babcock, J. M., Schlenz, M., Meade, T., Thompson, G. D., Bing, J. W., & Huckaba, R. M. (2010). Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. *Journal of Economic Entomology*, *103*(4), 1031–1038.

Trapnell, C., Roberts, A., Goff, L. A., Pertea, G., Kim, D., Kelley, D. R., … Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols*, *7*(3), 562–578. https://doi.org/10.1038/nprot.2012.016

Walker, B. J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., … Earl, A. M. (2014). Pilon: An integrated tool for comprehensive microbial variant detection and genome assembly im- provement. *PLoS One*, *9*, e112963. https://doi.org/10.1371/journal.pone.0112963

Wan, F., Yin, C., Tang, R., Chen, M., Wu, Q., Huang, C., … Wang, G. (2019). A chromosome-level genome assembly of *Cydia pomonella* provides insights into chemical ecology and insecticide resistance. *Nature Communications*, *10*(1), 1–14. https://doi.org/10.1038/s41467-019-12175-9

Westbrook, J. K., Nagoshi, R. N., Meagher, R. L., Fleischer, S. J., & Jairam, S. (2016). Modeling seasonal migration of fall armyworm moths. *International Journal of Biometeorology*, *60*(2), 255–267. https://doi.org/10.1007/s00484-015-1022-x

Williams, W. P., Buckley, P. M., & Daves, C. A. (2006). Identifying resistance in corn to southwestern corn borer (Lepidoptera: Crambidae), fall armyworm (Lepidoptera: Noctuidae), and corn earworm (Lepidoptera: Noctuidae). *Journal of Agricultural and Urban Entomology*, *23*, 87–95.

Wiseman, B. R., & Widstrom, N. W. (1984). Fall armyworm damage ratings on corn at various infestation levels and plant development stages. *Journal of Agricultural Entomology*, *1*(2), 115–119.

Xia, Q. Y., Wang, J., Zhou, Z. Y., Li, R. Q., Fan, W., Cheng, D. J., … Xiang, Z. H. (2008). The genome of a lepidopteran model insect, the silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology*, *38*(12), 1036–1045. https://doi.org/10.1016/j.ibmb.2008.11.004

Yang, F., Morsello, S., Head, G. P., Sansone, C., Huang, F., Gilreath, R. T., & Kerns, D. L. (2018). F2 screen, inheritance and cross-resistance of field-derived Vip3A resistance in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) collected from Louisiana, USA. *Pest Management Science*, *74*(8), 1769–1778.

Yang, J., Lee, S. H., Goddard, M. E., & Visscher, P. M. (2011). GCTA: A tool for genome-wide complex trait analysis. *The American Journal of Human Genetics*, *88*(1), 76–82.

You, M., Yue, Z., He, W., Yang, X., Yang, G., Xie, M., … Douglas, C. J. (2013). A heterozygous moth genome provides insights into herbivory and detoxification. *Nature Genetics*, *45*(2), 220–227. https://doi.org/10.1038/ng.2524

Yu, S. J. (1991). Insecticide resistance in the fall armyworm, *Spodoptera frugiperda* (J. E. Smith). *Pesticide Biochemistry and Physiology*, *39*(1), 84–91. https://doi.org/10.1016/0048-3575(91)90216-9

Yu, S. J., Nguyen, S. N., & Abo-Elghar, G. E. (2003). Biochemical characteristics of insecticide resistance in the fall armyworm, *Spodoptera*

frugiperda (J. E. Smith). *Pesticide Biochemistry and Physiology*, *77*(1), 1–11. https://doi.org/10.1016/S0048-3575(03)00079-8

Zhan, S., Merlin, C., Boore, J. L., & Reppert, S. M. (2011). The monarch butterfly genome yields insights into long-distance migration. *Cell*, *147*(5), 1171–1185. https://doi.org/10.1016/j.cell.2011.09.052

Zhang, L., Liu, B., Jiang, Y., Liu, J., Wu, K., & Xiao, Y. (2019). Molecular characterization analysis of fall armyworm populations in China. *Plant Protection*, *45*, 10–27.

Zhu, Y. C., Blanco, C. A., Portilla, M., Adamczyk, J., Luttrell, R., & Huang, F. (2015). Evidence of multiple/cross resistance to Bt and organophosphate insecticides in Puerto Rico population of the fall armyworm, *Spodoptera frugiperda*. *Pesticide Biochemistry and Physiology*, *122*, 15–21. https://doi.org/10.1016/j.pestbp.2015.01.007

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zhang L, Liu B, Zheng W, et al. Genetic structure and insecticide resistance characteristics of fall armyworm populations invading China. *Mol Ecol Resour*. 2020;20:1682–1696. https://doi.org/10.1111/1755-0998.13219

Received: 29 November 2019 | Revised: 27 May 2020 | Accepted: 6 July 2020

DOI: 10.1111/mec.15556

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Odorant binding proteins promote flight activity in the migratory insect, *Helicoverpa armigera*

Shang Wang[1,2] | Melissa Minter[2,3] | Rafael A. Homem[2] | Louise V. Michaelson[4] | Herbert Venthur[5,6] | Ka S. Lim[2] | Amy Withers[7] | Jinghui Xi[1] | Christopher M. Jones[2,8] | Jing-Jiang Zhou[1,2]

[1]College of Plant Sciences, Jilin University, Changchun, China

[2]Biointeractions and Crop Protection, Rothamsted Research, Harpenden, UK

[3]Department of Biology, University of York, York, UK

[4]Plant Sciences, Rothamsted Research, Harpenden, UK

[5]Laboratorio de Química Ecológica, Departamento de Ciencias Químicas y Recursos Naturales, Universidad de La Frontera, Temuco, Chile

[6]Centro de Investigación Biotecnológica Aplicada al Medio Ambiente (CIBAMA), Universidad de La Frontera, Temuco, Chile

[7]Lancaster Environment Centre, Lancaster University, Lancaster, UK

[8]Vector Biology Department, Liverpool School of Tropical Medicine, Liverpool, UK

Correspondence
Christopher M. Jones, Vector Biology Department, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK.
Email: chris.jones@lstmed.ac.uk

Jing-Jiang Zhou, College of Plant Sciences, Jilin University, Changchun, Jilin Province 130062, China.
Email: jjzhouchina@163.com

Funding information
Jilin University, Grant/Award Number: TAQ(JZ)-2017[7]-201811; Biotechnology and Biological Sciences Research Council, Grant/Award Number: BB/N012011/1 and BBS/E/C/000I0420

Abstract

Migratory insects are capable of actively sustaining powered flight for several hours. This extraordinary phenomenon requires a highly efficient transport system to cope with the energetic demands placed on the flight muscles. Here, we provide evidence that the role of the hydrophobic ligand binding of odorant binding proteins (OBPs) extends beyond their typical function in the olfactory system to support insect flight activity via lipid interactions. Transcriptomic and candidate gene analyses show that two phylogenetically clustered OBPs (OBP3/OBP6) are consistently over-expressed in adult moths of the migrant Old-World bollworm, *Helicoverpa armigera*, displaying sustained flight performance in flight activity bioassays. Tissue-specific over-expression of *OBP6* was observed in the antennae, wings and thorax in long-fliers of *H. armigera*. Transgenic *Drosophila* flies over-expressing an *H. armigera* transcript of OBP6 (HarmOBP6) in the flight muscle attained higher flight speeds on a modified tethered flight system. Quantification of lipid molecules using mass spectrometry showed a depletion of triacylglyerol and phospholipids in flown moths. Protein homology models built from the crystal structure of a fatty acid carrier protein identified the binding site of OBP3 and OBP6 for hydrophobic ligand binding with both proteins exhibiting a stronger average binding affinity with triacylglycerols and phospholipids compared with other groups of ligands. We propose that HarmOBP3 and HarmOBP6 contribute to the flight capacity of a globally invasive and highly migratory noctuid moth, and in doing so, extend the function of this group of proteins beyond their typical role as chemosensory proteins in insects.

KEYWORDS

Helicoverpa, insect migration, odorant binding proteins

1 | INTRODUCTION

Insect flight is one of the most energetically demanding processes in the animal kingdom. Long-distance insect migrants can actively sustain periods of flight for several hours. To achieve these remarkable feats of endurance, migratory insects have evolved a suite of morphological, sensory and physiologically traits that form part of an inherited "migratory syndrome" (Dingle, 2014; Liedvogel, Akesson, & Bensch, 2011; Roff & Fairbairn, 2007). Comparative genomics and quantitative trait analyses reveal considerable genetic variation for single migratory traits but the associated molecular genetic mechanisms and biochemical pathways remain poorly understood.

The vital role of chemical cues in host location and oviposition (Bruce & Pickett, 2011; Hansson & Stensmyr, 2011; Mescher & De Moraes, 2015) means that the involvement of a sophisticated olfactory system in migration and flight ability is an attractive proposition (Getahun et al., 2016; McCormick et al., 2017). For example, the odorant receptor family (OR), central to the olfactory system of pterygotes, emerged prior to the evolution of winged flight in insects as an adaptation to terrestrial life (Brand et al., 2016). New evidence suggests that OR-based signal transduction in *Drosophila* is necessary for efficient odour localization in flight (Getahun et al., 2016). Our recent transcriptomic work (RNA sequencing [RNA-seq]) in the Old World bollworm, *Helicoverpa armigera*, has shown that specific odorant binding proteins (OBPs), OBP3 and OBP6, are highly and consistently over-expressed in moths displaying sustained flight activity (Jones et al., 2015). This suggests that OBPs have a direct or indirect role in supporting insect flight and their function extends beyond their part in host-seeking and mating behaviour.

Insect OBPs are small, water-soluble extracellular transporter proteins (13–16 kDa; Lartigue et al., 2002; Tegoni, Campanacci, & Cambillau, 2004; Zhou, 2010), and possess extreme diversity between species with as little as 8% amino acid conservation (Pelosi, Zhou, Ban, & Calvello, 2006; Zhou, He, Pickett, & Field, 2008). They are generally thought to contribute to the sensitivity of the olfactory system by participating in the binding, solubilization and transportation of hydrophobic ligands through the sensillum lymph of the antennae (Grosse-Wilde, Svatos, & Krieger, 2006; Leal, 2013; Tsuchihara et al., 2005) and in protecting odours from enzymatic degradation (Chertemps et al., 2012; Gomez-Diaz, Reina, Cambillau, & Benton, 2013). Some OBPs, however, are found in nonchemosensory tissues and may participate in other physiological processes (Graham et al., 2001; Guo et al., 2011; Missbach, Vogel, Hansson, & Grosse-Wilde, 2015; Pelosi et al., 2006). The *Drosophila* OBP28a is not required for odorant transport and signal transduction, implying a different function altogether (Larter, Sun, & Carlson, 2016). The homologues of OBP6 and OBP3 in *H. armigera* are highly expressed in nonolfaction tissues in other noctuid moths, *Agrotis ipsilon* and *Helicoverpa assulta* (Gu et al., 2014; Li et al., 2015). In arthropods, OBPs are found exclusively in insects (Pelosi, Iovinella, Felicioli, & Dani, 2014) and comparative genomics suggests that the evolution of this protein family provided a mechanism to mediate the transport of hydrophobic chemical signals present in a terrestrial environment (Vieira & Rozas, 2011).

The Noctuidae family of moths possess some of the most important and polyphagous agricultural insect pests globally. A key characteristic that makes them such devastating pests is their ability to spread hundreds of kilometres in response to deteriorating local conditions. This exacerbates their potential to invade new territories, as observed with the current fall armyworm (*Spodoptera frugiperda*), which has spread eastwards into the Asian continent and the rapid expansion of *H. armigera* in the Americas following its recent incursion (Fitt, 1989; Jones, Parry, Tay, Reynolds, & Chapman, 2019). Adult moths from both species can climb to high altitudes and sustain active flight for several hours (Chapman et al., 2010). This requires an enormous amount of fuel consumption, metabolism and intracellular transport to the flight muscles. Given the well-established hydrophobic binding capacity of OBPs and their over-expression in *H. armigera*, it is possible that this group of proteins act as lipid transport carriers in *H. armigera*—the main flight fuel of migratory insects (Van der Horst & Ryan, 2012).

In the present study, we use a combination of behavioural, molecular, transgenic and protein modelling approaches to (a) determine the tissue-specificity of two OBPs consistently expressed in *H. armigera* adult moths demonstrating sustained flight activity, (b) show that the transgenic overexpression of one of these OBPs leads to enhanced flight performance in *Drosophila*, (c) identify the primary lipids depleted in *H. armigera* following flight and (d) identify the key residues responsible for lipid binding. Overall, our findings provide evidence that a subset of OBPs are responsible for binding key lipids commonly used by insect migrants and that this relationship promotes insect flight.

2 | MATERIALS AND METHODS

2.1 | *Helicoverpa armigera* strains

The adult *Helicoverpa armigera* used in this study originated from a long-term laboratory strain, *Bayer* (courtesy of the Max Planck Institute), and a wild-caught population from Spain (courtesy of the University of Valencia). The moths used in the RNA-seq were from a colony established from northern Greece. Insects were reared under a constant light regime of 14:10 hr light–dark at 26 ± 1°C in the insectaries of Rothamsted Research and the flight mill trials were conducted under the same conditions. Larvae were reared individually in 37-ml clear plastic pots containing a chickpea artificial diet and allowed to pupate before transfer to clean pots filled with vermiculite. Adult emergence was checked daily and any emerged individuals were set aside for flight mill trials or for rearing onto the next generation.

2.2 | Flight propensity of *H. armigera* measured by tethered flight mill

A series of flight mill experiments were conducted to determine the effects of over-expression of candidate genes associated with

migration or flight in *H. armigera* displaying contrasting flight abilities. A detailed description of the flight mill system is explained elsewhere (Jones et al., 2015; Minter et al., 2018). Insects from *Bayer* and *Spain* strains were reared through at least one generation in the insectary prior to flight mill trials and each strain was flown in independent experiments. Adult moths assigned to flight mill trials were placed at 4–10°C to facilitate the attachment of ~60-mg pins to the thorax with adhesive glue. Each moth was provided with 10% honey water solution ad libitum prior to flight. Moths were attached randomly to one of 16 flight mills via a pin and allowed to rest on a paper platform until the first flight was initiated by the insect. All flights took place between 7 p.m. and 9 a.m. under a 10-hr dark cycle from 8 p.m. to 6 a.m. At ~9 a.m. the next morning, individuals were taken off the mills and placed into individual pots for weighing. Any dead, unhealthy (e.g., broken wings or damage through improper handling) or escaped individuals were recorded and excluded from further analyses. All individuals were snap-frozen or placed in RNAlater within 2 hr and stored at −80°C for downstream molecular analysis.

The aggregated response variables were calculated for all individuals. We considered four response variables as being important discriminants of "strong" and "weak" fliers based on previous experiments; total distance flown (m), average speed flown (m/s), maximum speed attained (m/s) and number of bouts. Seven individuals from each strain were selected for RT-qPCR (reverse transcriptase quantitative polymerase chain reaction) of candidate genes from the ends of the flight activity distribution based on total distance and number of flight bouts.

2.3 | Tissue-specific candidate gene expression profiling in *H. armigera* flown on the flight mills

Initially, we determined the expression of 20 candidate genes from the head and thorax of 28 individual moths flown on the mills. The head (including the antennae) and thorax were removed using dissection instruments and placed in separate Eppendorf tubes with lysis buffer. The samples were homogenized using pellet pestles (Sigma-Aldrich). RNA was extracted using an Isolate II RNA Mini Kit (Bioline) and RNA was eluted in RNase-free water. cDNA was synthesized from 230 ng total RNA using SuperScript IV Reverse Transcriptase (Invitrogen) and Oligo$(dT)_{20}$ (Invitrogen).

Twenty candidate genes were screened for gene expression levels. qPCR primers were screened over a five-fold serial dilution of a cDNA sample (1/10th to 1/6,000th) and the primer efficiency was calculated. qPCRs were completed on the RotorGene 6000 (Qiagen) with conditions of 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 57°C for 15 s and 72°C for 20 s, followed by a melt curve analysis. Each reaction contained 10 μl of SYBR Green JumpStart Taq ready mix (Sigma-Aldrich), 300 nm of each primer and 5 μl of cDNA (1/50th dilution). The control genes *β-actin* and *elongation factor 1-α* were used for normalization (Wang, Dong, Desneux, & Niu, 2013; Yan et al., 2013) and all reactions were run in duplicate. Ct values were adjusted for primer pair efficiency. Expression levels were compared using a two-sided *t* test on the dCt values ($p < .05$) and are presented as $\log_{10}$ fold-change using ddCt (Schmittgen & Livak, 2008). RNA-seq was performed on moths flown and not-flown ($N = 4$ per group) according to previously described methods (Jones et al., 2015). All genes were considered significantly expressed at a false discovery rate of $p < .1$.

Following the identification of strong OBP expression profiles from the 20 candidate genes we determined the tissue-specific expression of OBP6 in the antennae, heads, thoraces, abdomens, legs and wings of *H. armigera* flown on the flight mills. Tissues were dissected from 18 adults and promptly immersed in liquid nitrogen and stored at −80°C. RNA was extracted using RNA-Solv reagent (Omega) following the manufacturer's protocol. Total RNA was quantified and checked for purity and integrity using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and gel electrophoresis. HiScript II Q RT SuperMix for qPCR with gDNA wiper (R223-01; Vazyme) was used for cDNA synthesis.

For tissue-specific expression profiling, RT-qPCR primer pairs were designed using PRIMER 5 software (Untergasser et al., 2012) and the same control genes used as above. mRNA levels were measured by RT-qPCR using the ChamQTM SYBR qPCR Master Mix (Vazyme). Each amplification reaction contained 1 μl synthesized cDNA, 10 μl of 2× ChamQTM SYBR qPCR Master Mix, 0.4 μl of 10 μm forward primer, 0.4 μl reverse primer and 8.2 μl water in a 20-μl reaction mix. Reactions were performed on an ABI 7500 Real-Time PCR System (Applied Biosystems) under the following conditions: 30 s denaturation at 95°C and 40 cycles of 95°C for 10 s and 60°C for 30 s, followed by a melt curve for specificity analysis. Fold-change values were calculated from the mean of three biological replicates with the ddCt method and using the abdomen as the calibrator. Relative expression levels were compared using the dCt values ($p < .05$) as described above.

2.4 | Quantitative triacylglycerol analysis

Total lipids were extracted from moth tissue ground in liquid nitrogen (Usher et al., 2017). The molecular species of triacylglycerols (TAGs) were analysed by electrospray ionization triple quadrupole mass spectrometry (ESI-MS; using API 4000 QTRAP; Applied Biosystems). TAGs are defined by the presence of one acyl fragment and the mass/charge of the ion formed from the intact lipid (neutral loss profiling; Krank, Murphy, Barkley, Duchoslav, & McAnoy, 2007). This allowed identification of one TAG acyl species and the total acyl carbons and total number of acyl double bonds in the other two chains. The procedure does not allow identification of the other two fatty acids individually nor the positions (sn-1, sn-2 or sn-3) that individual acyl chains occupy on the glycerol. TAG was quantified after background subtraction, smoothing, integration, isotope deconvolution and comparison of sample peaks with those of the internal standard (using LipidView, AB-Sciex). The profiling samples were prepared by combining 10 μl of the total lipid extract with 990 μl of isopropanol/methanol/50 mm ammonium acetate/dichloromethane (4:3:2:1). Samples

were infused at 15 μl/min with an autosampler (LC mini PAL, CTC Analytics). The scan speed was 100 μ s^{-1}. The collision energy, with nitrogen in the collision cell, was set to +25 V; declustering potential +100 V; entrance potential 14 V; and exit potential +14 V. Sixty continuum scans were averaged in the multiple channel analyser mode. For product ion analysis, the first quadrupole mass spectrometer (Q1) was set to select the TAG mass and Q3 for the detection of fragments produced by collision-induced dissociation. The mass spectral responses of various TAG species are variable, owing to differential ionization of individual molecular TAG species. For all analyses gas pressure was set on "low," and the mass analysers were adjusted to a resolution of 0.7 μm full width height. The source temperature was set to 100°C, the interface heater was deployed, +5.5 kV applied to the electrospray capillary; the curtain gas was set at 20 (arbitrary units); and the two ion source gases were set at 45 (arbitrary units). The data were normalized to the internal standard Tri15:0 (Sigma Aldrich) and further normalized to the weight of the initial sample.

2.5 | Quantitative phospholipid analysis

Quantitative analyses to measure phospholipids (PL), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylserine (PS) were carried out using electrospray ionization tandem triple-quadrupole mass spectrometry (API 4000 QTRAP; Applied Biosystems; ESI-MS/MS). The lipid extracts were diluted and resuspended in $CHCl_3$/MeOH/300 mm ammonium acetate in water, 300:665:35. Internal standards were obtained and quantified as previously described (Devaiah et al., 2006). Samples were directly infused at 15 μl/min with an autosampler (HTS-xt PAL, CTC-PAL Analytics). Data acquisition and acyl group identification of the polar lipids was performed, with modifications, from Ruiz-Lopez, Haslam, Napier, and Sayanova (2014). The internal standards were supplied by Avanti, incorporated as 0.085 nmol di24:1-PC, 0.08 nmol di14:0-PE, 0.08 nmol di18:0-PI, 0.032 nmol di18:0-PS and 0.08 nmol di14:0-PG.

The molecular species of polar lipids were defined on the basis of the presence of a head-group fragment and the mass/charge of the intact lipid ion formed by ESI. However, tandem ESI-MS/MS precursor and product ion scanning, based on head group fragment, did not determine the individual fatty acyl species. Instead, polar lipids were identified at the level of class, total acyl carbons and total number of acyl carbon–carbon double bonds.

The data were processed using the program LIPID VIEW SOFTWARE (AB-Sciex) where isotope corrections are applied. The peak area of each lipid was normalized to the internal standard and further normalized to the weight of the initial sample. A parametric two-sided *t* test was used to compare lipid content between flown and not flown moths ($N = 4–5$ moths per group).

2.6 | Phylogenetic analysis of *H. armigera* OBPs

N-terminal signal peptides of OBPs were predicted by signal ip 4.0 (http://www.cbs.dtu.dk/services/SignalP/). Alignment of amino acid sequences (without signal peptides) was performed by mafft (https://www.ebi.ac.uk/Tools/msa/mafft/). The phylogenetic trees of OBPs were constructed using MEGA6 software by the maximum-likelihood method with 1,000 bootstraps with the *p*-distance model (Gong, Zhang, Zhao, Xia, & Xiang, 2009).

2.7 | Development of a novel flight mill for *Drosophila melanogaster* and other small insects

We designed a new set of flight mills to accommodate smaller insects to examine the flight ability of wild type and transgenic *Drosophila* flies (Figure S1). These flight mills are similar in structural design to those used in the *H. armigera* experiments, comprising a flight arm and rotational disc to allow flies to move around an axis by means of a very low-friction magnetic bearing (Figure S1).

As part of the study we developed a robust standard operating procedure for tethering *Drosophila*. Briefly, an individual fly was lightly anaesthetized with CO_2 and transferred to a custom-made paper groove which had been made to allow accurate positioning of an anaesthetized fly (Figure S1). The paper groove was placed on the platform with CO_2 passing through the groove bottom. When the flies were under CO_2 anaesthesia, the tip of a small flight mill arm (a 5-cm-long, 0.2-mm-diameter copper wire) was tethered onto the dorsal side of the anaesthetized fly's thorax with Super Glue under a stereomicroscope (Olympus SZ40). Individual flies and the small flight mill arms were gently handled with either a small brush or jeweller's vacuum tweezers. Once the glue was dry and hard, the tethered flies were moved to the experimental chamber, fed with sucrose solution from a small piece of filter paper and allowed to rest in the recording chambers to adapt to the experimental environment overnight prior to data collection. At 10 a.m. the following day, filter papers were removed from the recording chambers and data collection was started using the same software as the larger mills. Experiments were run until ~2.30 p.m. to ensure each mill had run for at least 3 hr. Any flies which looked damaged, unhealthy or had escaped from the flight arm were disregarded from further analyses.

2.8 | Generation of transgenic *Drosophila* expressing *HarmOBP6*

All *Drosophila* strains were maintained on standard food (Bloomington formulation) at 24°C and 65% relative humidity on a 12/12-hr light/dark cycle. Proteinase K treatment and phenol/chloroform extraction were used to isolate genomic DNA (gDNA) from adult *D. melanogaster* flies for use in PCR.

HarmOBP6 (B5X24_HaOG200803 with the addition of a stop codon) was codon-optimized for expression in *D. melanogaster* and synthetized by GeneArt (ThermoFisher Scientific). The codon-optimized sequence was transferred from the subcloning plasmid pMA (GeneArt) to the attB-carrying plasmid pUAST (pUASTattB_EF362409) using restriction enzymes *Eco*RI and *Xho*I. The pUAST-*Harm-OBP6* construct was microinjected into syncytial blastoderm

embryos of an integration strain (y w M[eGFP, vas-int, dmRFP]ZH-2A; Pattp40; Dundas et al., 2006) that carries an *attP* docking site on the second chromosome (*attP40*) and the *phiC31* integrase gene under control of a germline-specific (*vasa*) promoter on the X chromosome. This strain was sourced from the Fly Facility, University of Cambridge. The GAL4 strain (w[1118]; Pw[+mW.hs] = GawBDJ757) was sourced from Bloomington Drosophila Stock Centre (BDSC-8184). Microinjections were performed in-house using an inverted microscope (eclipse TieU Nikon) equipped with a 10×/0.25 lens, 10×/22 eyepiece and fluorescence illumination. Injection mixtures consisted of 0.5× phosphate buffer (pH 6.8, 0.05 mm sodium phosphate, 2.5 mm KCl), 300 ng/μl of the pUAST-*Harm-OBP6* construct and 200 mg/L fluorescein sodium salt and delivered by a FemtoJet express micro-injector (Eppendorf) controlled by a motorized micromanipulator (TransferMan NK2; Eppendorf). Injection needles were prepared following Miller, Holtzman, and Kaufman (2002).

Micro-injection survivors were back-crossed and the F_1 progeny was screened for the presence of the *white* marker gene (orange eye phenotype). Positive flies were intercrossed to generate homozygous flies (red eyes) which were selected to establish the final strain. Control flies carrying an empty pUAST plasmid (*UAS-empty* strain) were generated following the same protocols described above.

2.9 | Tethered flight of transgenic *Drosophila* and statistical analysis of flight response variables

Three flight mill experiments were performed to compare the flight ability of transgenic *Drosophila* flies carrying *HarmOBP6* (GAL4 > UAS-OBP6 line) with control flies (GAL4 > UAS line). In addition, we were also interested in how flight activity changes with the age of the fly. A total of eight flies were flown simultaneously per run with each trial consisting of a mixture of *HarmOBP* and control flies.

1. Experiment 1: flies generated from crosses between male *UAS-OBP6* (*UAS-empty* for controls) and female *muscle-GAL4* strains. *GAL4 > UAS-OBP6* virgin females ($N = 28$) were flown on the mills alongside *GAL4 > UAS* virgin female control flies ($N = 28$). The age of the flies in this experiment ranged from 24 to 144 hr after emergence.
2. Experiment 2: flies were generated from crosses between female *UAS-OBP6* (*UAS-empty* for controls) and male *muscle-GAL4*. Both *GAL4 > UAS-OBP6* ($N = 23$) and *GAL4 > UAS* control ($N = 21$) female flies were mated prior to the flight mill trials. Flies were either 2, 6 or 15 days old after emergence.
3. Experiment 3: *GAL4 > UAS-OBP6* ($N = 43$) and *GAL4 > UAS* ($N = 34$) control flies were generated as in Experiment 2 but without mating. The age of the flies ranged from 7 to 26 days old after emergence.

After preliminary trials, we determined that a 1-hr cut-off period was sufficient to measure flight performance with difference between the average speeds attained between 1 and 3 hr of flight (example from Experiment 3 in Figure S2). We were primarily interested in the two response variables, the average speed flown (AVGSP; m/s) and maximum speed attained (MAXSP; m/s). We hypothesized that the average or maximum speed of flight is a much more useful metric to distinguish the flight activity of flies such as *Drosophila* because they are not capable of sustaining hours of flight like larger insects (e.g., Lepidoptera). The distribution of each flight parameter was assessed using the FITDIST package (Delignette-Muller & Dutang, 2015) using QQ plots and goodness of fit statistics. AVGSP and MAXSP were both normally distributed (Figure S2).

Data were fitted using generalized linear mixed models (GLMMs) using the LME4 package in R (Bates, Machler, Bolker, & Walker, 2015). To model AVGSP and MAXSP as a function of the covariates we used a Gaussian linear mixed-effects model. Fixed covariates were *strain* (transgenic or control) and *age* (categorical). An interaction term *strain* × *age* was included. To incorporate differences between the flight mills on which the individual was flown we included *mill* as a random effect. Best-fit GLMMs were created using a backward stepwise approach from the maximally complex model, which included the interaction. Explanatory variables were retained in the best-fit model according to significance ($p < .05$) in likelihood ratio tests (LRTs). Model assumptions were verified using residual-fitted plots. Predictions of response variables from each model were made using least square means (LSMs) in the package LSMEANS (Lenth, 2016) and differences between groups assessed using Tukey post hoc tests.

2.10 | Homology structure modelling of *H. armigera* OBPs

The amino acid sequences of HarmOBP6 and HarmOBP3 were used as a target while the template was the crystal structure of the blowfly *Phormia regina* OBP56a (PregOBP56a) (PDB code: 5DIC). The pheromone binding protein 1 from the silkmoth *Bonbyx mori* BmorPBP1 (1DQE) was used as the template for HarmPBP1 structure modelling. Five hundred models of each OBP were obtained using MODELLER9.14 (http://salilab.org/modeller) and the best initial model was selected according to the lowest discrete optimized protein energy (DOPE) score provided by the software. The stereochemistry of the best model was assessed using the theoretical validation package PROCHECK (Laskowski, Macarthur, Moss, & Thornton, 1993), and the overall structure was visualized using PYMOL software (http://www.pymol.org). Further refinement steps were carried out with NAMD version 2.9 (parallel molecular dynamics code for biomolecular system simulation) installed in the high-performance computer (HPC) Lautaro Linux cluster at Centro de Modelación y Computación Científica (CMCC) from Universidad de La Frontera. The CHARMM36 (Huang et al., 2016) force field was used for all the simulations. The selected protein model was solved with the TIP3P water model in a cubic box with a minimum distance of 10 Å between the protein and the edge of the box. Neutralization of the protein–water system was performed by adding Na^+ or Cl^- randomly placed in the box. Likewise, the system was simulated under periodic boundary conditions with a cutoff radius of 12 Å for nonbonded interactions and a time step of 2 fs. Alpha-carbons (Cα) of secondary structures were fixed with a constant force of 1 kcal/mol $Å^{-1}$. A first energy minimization of 10,000

steps was performed followed by heating through short simulations of 1 ps at 50, 100, 150, 200, 250 and 300 K. Long simulations were kept at 300 K and 1 bar pressure in the NTP (referred to a constant number of particles, temperature and pressure) over 50 ns. A root-mean-square deviation (RMSD) trajectory tool was used to calculate the RMSD with reference to the starting structure (Figure S3). Therefore, when the plotted RMSD showed small fluctuations (~1–1.5 Å), coordinates were analysed by PROCHECK every 100 frames to obtain the best structure (lowest energy). Finally, the putative binding site and its volume were calculated via the CASTp server (http://sts-fw.bioengr.uic.edu/castp/calculation.php; Dundas et al., 2006).

2.11 | Molecular docking

The refined structures of HarmOBP6 and HarmOBP3 were used as the target for molecular docking with AUTODOCK VINA (Trott & Olson, 2010). Likewise, a refined 3D structure of the pheromone binding protein HarmPBP1 was used as the reference template for the molecular docking tasks based on its reported function in binding sex pheromones (Dong et al., 2017; Ye et al., 2017). Energy minimization and optimization for the ligands used in this study were performed using MM2 minimization methods in the CHEM3D 16.0 Software (Perkin Elmer). For HarmOBP6, polar hydrogens were added using the interface AUTODOCK TOOLS, as well as torsional bonds for ligands. A grid box with $26 \times 26 \times 26$ points and a default space of 1 Å was prepared via AUTOGRID following the predicted binding site by the CASTp server. For every docking run, an exhaustiveness of 500 was considered and the best binding modes were selected according to the lowest free binding energy (kcal/mol). The TAGs and phospholipids were energy-minimized following the same protocol for fatty acids and semiochemicals. Considering that AUTODOCK VINA allows a maximum of 32 rotatable bonds, these compounds and their binding to HarmOBP6 and HarmOBP3 were submitted to the DINC server (http://dinc.kavrakilab.org/; Antunes et al., 2017; Dhanik, McMurray, & Kavraki, 2013). This server was used to dock the lipids into the HarmOBP6 and HarmOBP3 binding site following the above grid box parameters and with all rotatable bonds active. The DINC server allows docking for large molecules based on the AUTODOCK algorithm and fragmentation processes, for which fragments that show best binding are incrementally expanded by adding atoms of the ligand to it in each of several rounds. Thus, both fully flexible and bound conformations of lipid molecules were extracted and docked again into HarmOBP6, HarmOBP3 and HarmPBP1 (control) using AUTODOCK VINA.

3 | RESULTS AND DISCUSSION

3.1 | Two *Helicoverpa armigera*-specific OBPs are overexpressed in the thorax of moths displaying prolonged flight activity

Adult moths from two colonized strains of *Helicoverpa armigera* (*Bayer* and *Spain*) were flown overnight on a computerized tethered flight mill system that experimentally quantifies the flight performance of individual insects in the absence of external stimuli (Minter et al., 2018). Previous flight mill studies with noctuid moths have shown an inverse relationship between the total distance flown and the number of individual flight bursts to discriminate those insects engaging in prolonged or more appetitive behaviour (Jones et al., 2015). We used this relationship to assign individual moths into two distinct flight activity groups, "short-distance" (SD) or "long-distance" (LD), for downstream gene expression analyses (Figure 1a).

We undertook a candidate gene approach to determine the differential expression of 20 genes in the two strains of *H. armigera* flown on the flight mills. As a baseline control, and to validate some of our previously detected candidate genes from whole transcriptome studies (Jones et al., 2015), RNA-seq of moths flown and not flown on the flight mills was performed. Eight of our 20 candidate genes were significantly up-regulated in the flown group with OBP6 showing the highest and most consistent level of up-regulation (Figure S4). Each gene has a reported role in insect migration or sustained flight activity including those involved in circadian and photoreceptor processes (Reppert, Guerra, & Merlin, 2016), lipid metabolism (Arrese & Soulages, 2010), OBPs (Jones et al., 2015), flight muscle structure (Zhan et al., 2014), and the metabolism of proline and phenylalaine/tryptophan (Arrese & Soulages, 2010; Rio, Attardo, & Weiss, 2016; Figure 1b).

In the *Bayer* strain four genes were significantly over-expressed in the thorax of the LD moths, all of which were up-regulated exclusively in the thorax and not the head (Figure 1b; Table S1). These four genes encode two OBPs (*OBP3* and *OBP6*), the *protein henna-like isoform X3* and a *fatty-acid synthase-like* gene. In the thorax of individuals from the strain *Spain*, three genes were significantly over-expressed in the LD group; *myofilin*, *OBP3* and *protein henna-like isoform X3*; and three genes were significantly over-expressed in the SD group, *collagen alpha subunit-1(IV)*, *cry-1* and *phospholipase A2-like*. Two genes were differentially expressed in the head of *H. armigera* individuals from the *Spain* strain (although the magnitude of this expression was small; Figure 1b).

Following the detection of OBP over-expression both in this study and from transcriptome profile analysis (RNA-seq [Figure S4]; Jones et al., 2015), we showed that the relative expression levels of *OBP3* and *OBP6* in individual *H. armigera* displayed a significant positive correlation with flight performance which was strongest in the thorax (*HarmOBP3*: head: $R = 0.49$, $p = .006$, thorax: $R = 0.81$, $p < .001$; *HarmOBP6*: head: $R = 0.31$, $p = .18$, thorax: $R = 0.65$, $p = .002$; Figure 1c). Furthermore, we quantified the expression of *HarmOBP6* in the antenna, head, thorax, abdomen, leg and wing of SD and LD moths. *HarmOBP6* was significantly over-expressed in the antennae ($p = .016$), thoraces ($p = .009$) and wings ($p = .05$) and this expression was significantly up-regulated in LD moths compared with those in the SD group (Figure 1d; Table S2).

The simple phenotypic comparisons of SD and LD insects presented here provide a measurement of flight performance in terms of the raw physiological capacity to fly. We recognize that a full spectrum of flight behaviours exists and that these are controlled by intricate internal and external processes. For example, the migratory flight behaviour of the Monarch butterfly (*Danaus plexippus*)

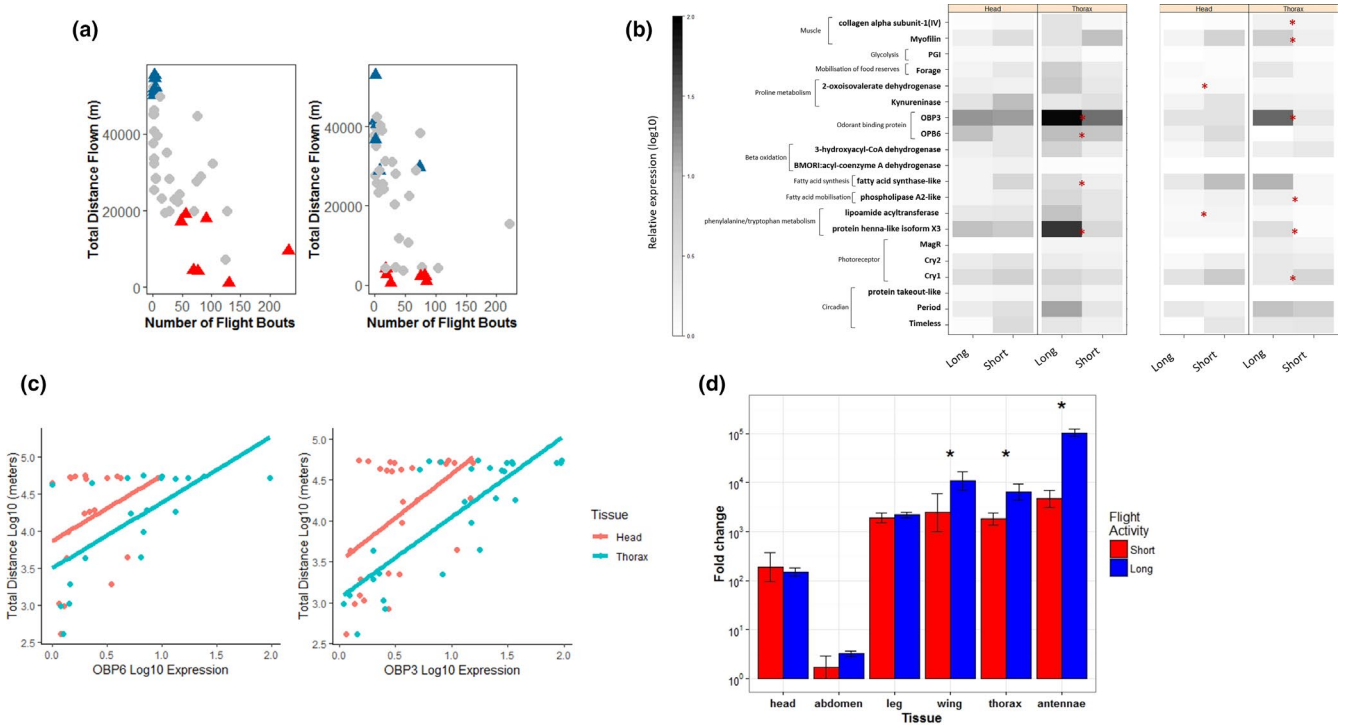


FIGURE 1 The expression of OBPs in adult *H. armigera* flown on tethered flight mills. (a) Flight activity of *H. armigera* moths characterized using tethered flight. Total distance flown and the number of flight bouts were used to discriminate moths displaying short-distance (red) or long-distance (blue) activity in two colonised strains, Bayer (left) and Spain (right). Individuals assigned to gene expression experiments denoted with a triangle. (b) A heatmap showing the RT-qPCR expression (log10) of twenty genes previously associated with migration and/or sustained flight activity in insects. Differential expression was determined between 'long' or 'short' fliers from the Bayer (left) and Spain strain (right). Genes are grouped by known physiological function. Expression levels were compared using a two-sided test on dCT values in the head and thorax and significance is denoted with the red * (non-adjusted $p < .05$). (c) The increase in OBP expression with flight performance measured by total distance flown (log10 metres). (d) Fold-change expression of OBP6 in six tissues from *H. armigera* flown on the flight mills. * represents significant differences in expression levels between flight groups ($p < .05$)

is controlled in response to environmental changes (temperature, photoperiod) via internal genetic, and possibly epigenetic, cascades (Merlin & Liedvogel, 2019). Here we focus on raw flight capacity and use our expression profiling to speculate on the functional role of OBPs in insect flight.

3.2 | Phylogenetic analysis of the odorant binding proteins implicated in *H. armigera* flight

An alignment of the protein sequences of HarmOBP3 (accession no.: AEB54582) and HarmOBP6 (accession no.: AEB54587) is provided in Figure S5. Based on the sequence alignment, HarmOBP6 belongs to the classic OBP subgroup, which contains typical characteristic sequence features of six conserved cysteine residues and the classic insect OBP motif: C_1-X_{15-39}-C_2-X_3-C_3-X_{21-44}-C_4-X_{7-12}-C_5-X_8-C_6 (Figure S5; Zhou et al., 2008). Phylogenetic analysis shows that HarmOBP3 and HarmOBP6 are clustered into the same branch with 100% bootstrap support between OBP3 and OBP6, indicating that they share a high homologous amino acid sequence similarity and probably a similar function (Figure S6). There is 81% amino acid identity between OBP3 and OBP6. HarmOBP6 is also closely clustered with other *Helicoverpa* OBPs such as *Helicoverpa assulta*, HassOBP6 (accession no.: AEX07270) and *Heliothis virescens*, HvirOBP0136 (accession no.: ACX53819; Figure S6).

3.3 | Transgenic *Drosophila* expressing OBP6 in the flight muscle attain higher speeds on a novel flight mill system

To functionally validate the role of OBPs in flight activity we generated a transgenic *D. melanogaster* strain that over-expresses *HarmOBP6* in muscle cells and assessed the performance of these flies on a newly designed flight mill system for small dipterans (Figure S1). We chose OBP6 based on its magnitude of expression in a Chinese strain of *H. armigera* previously reported as well as the flown/not flown comparison (Figure S4) but postulate that the high conservation between the protein sequences of OBP6 and OBP3 (see phylogenetic analysis above) would lead to similar results had we chosen OBP3. Transgenic strains were generated using the φC31 integration system (Bischof, Maeda, Hediger, Karch, & Basler, 2007). Genomic integration of *HarmOBP6* in generated transgenic flies, hereafter referred to as *UAS-OBP6* strain, was confirmed by PCR and sequencing (Figure 2a). The *GAL4/UAS* expression system (Brand & Perrimon, 1993) was used to induce the expression of *HarmOBP6* in

muscle cells by using a muscle-specific GAL4 driver strain (Seroude, Brummel, Kapahi, & Benzer, 2002) (referred to as *muscle-GAL4* strain). The over-expression of *HarmOBP6* in transgenic flies generated from the cross between the *UAS-OBP6* and *muscle-GAL4* strains (GAL4 > UAS-OBP6 flies) was confirmed by RT-PCR and RT-qPCR (Figure 2a). The expression of *HarmOBP6* increased by more than 15 times in *GAL4 > UAS-OBP6* flies when compared to parental *muscle-GAL4* and *UAS-OBP6* (Figure 2a).

We performed a series of flight mill experiments with three separate experimental trials. First, we compared the 1-hr flight activity of *Gal4 > UAS-OBP6* transgenic and *Gal4 > UAS* control flies. These flies are genetically identical, the only difference being the absence of *HarmOBP6* in the controls. The average and maximum speeds (m/s) attained during the 1 hr of flight activity were analysed using GLMMs as a function of the covariates strain and age (Table 1). There was no difference in the average or maximum speed between *Gal4 > UAS-OBP6* and *Gal4 > UAS* control flies when *Gal4 > UAS-OBP6* originated from crosses using *UAS-OBP6* as the male parent (Experiment 1, Table 1). In this experiment there was evidence for increased speeds in older (over 48 hr old) *Gal4 > UAS-OBP6* flies.

By contrast, *Gal4 > UAS-OBP6* flies originating from the reciprocal cross (*UAS-OBP6* as the female parent) flew consistently faster and attained higher maximum speeds than control flies (Figure 2b–e; Table 1) and this pattern was observed in both mated and virgin F_1 flies (Experiments 2 and 3). There was an effect of age in both experiments: flies from the older age groups (those flies emerging after 1 week) flew faster than the younger cohort. The discrepancy in the F_1 flight activity results between *UAS-OBP6* male and female parental lines could be due to maternal effects as observed in laboratory crosses of "short" and "long" flight phenotypes from other moth species (Gu & Danthanarayana, 1992).

3.4 | Quantification of TAG and phospholipids in flown *H. armigera*

We hypothesize that OBPs function as a fuel carrier for the supply of lipids to the flight muscles during prolonged flight in *H. armigera*. To determine candidate lipid molecular species for binding with OBPs we compared the total lipid content of age-matched moths flown on the flight mills with those reared to adults and not forced to undergo flight. Six lipid classes were assayed using ESI-MS including TAG, PE, PS, PI, PG and PC.

Unsurprisingly total TAG levels were (a) the most abundant class of the lipids analysed and (b) underwent the most pronounced decline in flown moths (1.8-fold reduction from 739.6 to 410.5 nmol/g fresh weight, $p = .006$; Figure 3a). Sustained flight activity in insects is powered primarily by the mobilization of TAG in the insect fat body into diacylglycerol (DAG), which is then shuttled in the haemolymph to the flight muscle (Van der Horst & Ryan, 2012).

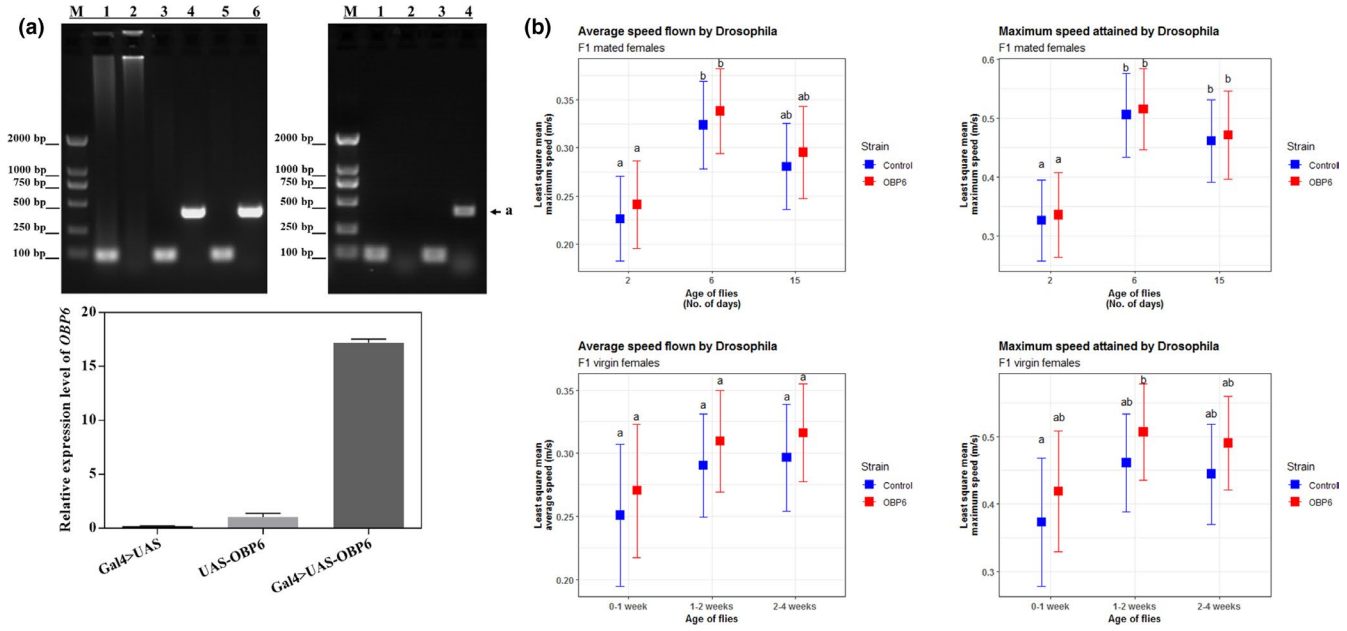


FIGURE 2 Effect of expression of *HarmOBP6* on Drosophila flight activity. (a) Genomic integration of HarmOBP6 into transgenic flies. (top panel) PCR products amplified from cDNA of transgenic flies. Lane 1, Rpl32 primers, control *GAL4>UAS* flies; Lane 2, OBP6 primers, control *GAL4>UAS* flies; Lane 3, Rpl32 primers, *GAL4>UAS-OBP6* female flies; Lane 4, OBP6 primers, *GAL4>UAS-OBP6* female flies; Lane 5, Rpl32 primers, *GAL4>UAS-OBP6* male flies; Lane 6, OBP6 primers, *GAL4>UAS-OBP6* male flies; M, DNA Maker. Symbol a indicates OBP6. (bottom panel) RT-qPCR of HarmOBP6 in *GAL4>UAS-OBP6* transgenic flies in comparison to GAL4>UAS and UAS-OBP6 control flies. (b) Flight activity bioassays with Drosophila expressing *HarmOBP6* with F1 virgin females (bottom row) and F1 mated feamles (top row). The average speed and maximum speed between transgenic and control Drosophila flies predicted using Least Square Means (LSMs) and differences between groups assessed using Tukey *post-hoc* tests. Results presented are for Experiment 2 (top row) and 3 (bottom row) with *Gal4>UAS-OBP6* flies originating from the reciprocal cross (*UAS-OBP6* as the female parent)

TABLE 1 Estimated regression parameters, standard errors and *t* values for GLMMs for *Drosophila* flight mill experiments

Experiment	Response	Regression parameter	Estimate	*SE*	*t* value
No. 1 F_0 = ♂*UAS-OBP6* × ♀*muscle-GAL4*	AVGSP	Intercept	0.316	0.021	14.70
F_1 = Virgin ♀		StrainOBP6	−0.019	0.026	−0.72
		AgeOver48h	0.048	0.026	1.84
	MAXSP	Intercept	0.481	0.033	12.35
		StrainOBP6	−0.017	0.035	−0.49
		AgeOver48h	0.067	0.037	1.84
No. 2 F_0 = ♀*UAS-OBP6* × ♂*muscle-GAL4*	AVGSP	Intercept	0.226	0.015	14.76
F_1 = Mated ♀		StrainOBP6	0.015	0.016	0.93
		Age6D	0.097	0.019	5.11
		Age15D	0.054	0.019	2.81
	MAXSP	Intercept	0.326	0.024	13.68
		StrainOBP6	0.010	0.025	0.40
		Age6D	0.179	0.030	6.05
		Age15D	0.135	0.030	4.50
No. 3 F_0 = ♀*UAS-OBP6* × ♂*muscle-GAL4*	AVGSP	Intercept	0.251	0.020	12.50
F_1 = Virgin ♀		StrainOBP6	0.019	0.015	1.34
		Age2weeks	0.039	0.021	1.90
		Age4weeks	0.046	0.021	2.22
	MAXSP	Intercept	0.373	0.034	11.03
		StrainOBP6	0.046	0.024	1.95
		Age2weeks	0.088	0.034	2.62
		Age4weeks	0.071	0.033	2.14

Note: AVGSP is the average speed and MAXSP is the maximum speed flown on the flight mill.

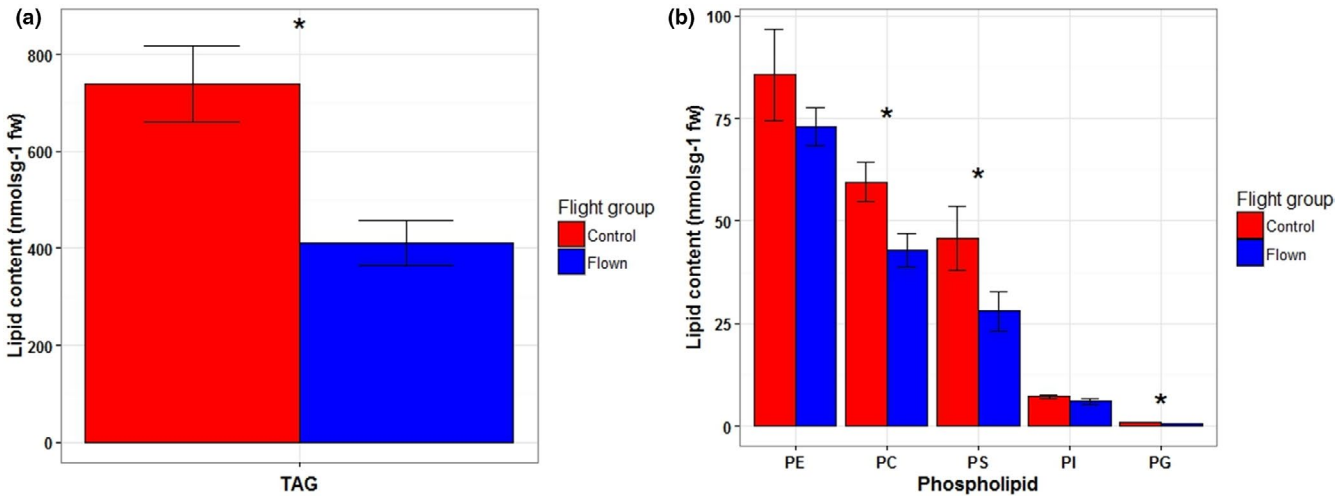


FIGURE 3 Lipid depletion in flown *H. armigera* measured by electrospray ionization tandem triple-quadrupole mass spectrometry (ESI-MS). (a) Total triacylglycerol (TAG) (nmolesg-1 per fresh weight (FW) content in flown versus control *H. armigera*. (b) Depletion of five phospholipid classes in flown moths; phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylcholines (PC). Error bars represent SE (n = 4-5 per group) and * indicates significant differences ($p < .05$) between flight groups

The role of phospholipid metabolism in insects is far less well understood although in this experiment we show a consistent depletion in each phospholipid class following flight (Figure 3b; see Figure S7 for individual lipid species and Table S3 for test statistics). In mammals the relative abundance of the two most common phospholipids, PC and PE (also the two most abundant classes in *H. armigera* moths measured by ESI-MS, Figure 3b), regulates the size and dynamics of lipid droplets and energy metabolism (Veen

et al., 2017). Phospholipids are critical to membrane structure and function; the fatty acyl components of the phospholipids can provide another potential energy source. When cells are subject to starvation, levels of phospholipid classes decrease (Steinhauser et al., 2018). Lipid droplets are storage organelles at the centre of lipid and energy homeostasis. They have a unique architecture consisting of a hydrophobic core of neutral lipids which is dominated by TAG, enclosed by a phospholipid monolayer that is decorated by a specific set of proteins (Olzmann & Carvalho, 2019). Utilizing the reserves of TAG from lipid droplets for energy will release phospholipids, which can also be metabolized.

3.5 | The protein structure and lipid binding site of *H. armigera* OBPs

We used the 3D structure of an OBP from the blowfly, *Phormia regina* (PregOBP56a; Ishida, Ishibashi, & Leal, 2013), as a template for HarmOBP6 and HarmOBP3, and the pheromone binding protein from the silkmoth *Bombyx mori* BmorPBP1 (1DQE) as a template for HarmPBP1, to build structural models and predict the binding efficacy to a range of fatty acids. We used HarmPBP1 as a positive control for semiochemical binding in relation to its observed role in female sex pheromone response (Ye et al., 2017). As expected, the structures of both HarmOBP6 and HarmOBP3 resemble typical insect OBPs, consisting of six α-helices connected by loops and three disulphide bridges that contribute to overall structural stability (Figures 4a and S8). Binding site prediction indicates the OBP has a pocket of 772.8 $Å^3$ volume and 917.1 $Å^2$ area for OBP6 and 777.9 $Å^3$ volume and 642.4 $Å^2$ area for OBP3 with a "Tunnel" conformation suitable for lipid binding (Figures 4b and S8).

To quantify the strength of molecular interactions between the over-expressed OBPs and potential substrates, molecular docking was conducted to determine binding energies with a range of fatty acids and olfactory odorants (semiochemicals; Table 2). A total of 33 compounds were selected to dock with the predicted HarmOBP6, HarmOBP3 and HarmPBP1 protein structures, including nine fatty acids, 15 semiochemicals (identified from the Pherobase database https://www.pherobase.com), L-proline (amino acid; Rio et al., 2016), D-trelahose (sugar) and a selection of DAG/TAG/phospholipid species analysed by ESI-MS (Table 2).

The *H. armigera* OBPs possessed the lowest overall binding energies with TAG and phospholipids (Table 2). Apart from PE, HarmOBP3 had a greater binding affinity to each long-chain lipid than OBP6, with mean docking values for modelled HarmOBP6 and HarmOBP3 of −16.30 ± 0.80 and −18.20 ± 1.84 kcal/mol respectively. The lowest values were observed for HarmOBP3:TAG (52:2) and HarmOBP:phosphatidylinositol (PI 36:3; Table 2). The predicted binding model for PI in the pocket of OBP3 and OBP6 is shown in Figure 4c) with optimal predictions for TAG and other phospholipids in Figure S8. In contrast, the binding predictions between HarmPBP1 and lipid molecules were highly inconsistent (Table 2). As expected from its putative role in sex pheromone transportation (Ye et al., 2017), HarmPBP1 bound semiochemicals and fatty acids with greater negative values compared to the OBPs (Table 2). There was little difference in semiochemical or fatty acid docking values between OBP6 and OBP3. Overall, these molecular docking patterns support the hypothesis that the *H. armigera* OBPs investigated in

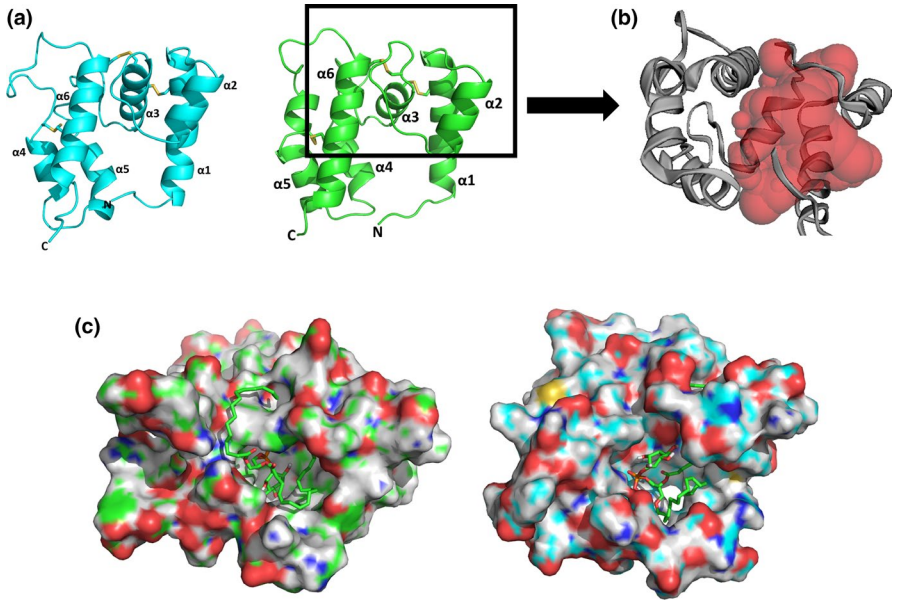


FIGURE 4 Predicted 3D-homolgy models of *H. armigera* OBPs and optimised docking predictions for phosphatidylinositol. (a) Protein helices for OBP3 (cyan) and OBP6 (green) are shown with disulfide bridges indicated by yellow sticks. C- and N-termini are highlighted with C and N, respectively. Alpha-helix domains are highlighted with "α" and corresponding numbers. (b) The binding site prediction of OBP6 using CASTp. Red surface indicates the pocket with 772.8 Å3 of volume and 917.1 Å2 of area. (c) Docking prediction of phosphatidylinositol (PI) with OBP3 (left) and OBP6 (right). This phospholipid had the lowest estimated free-energy of binding with both OBPs from AutoDock

TABLE 2 Molecular docking between HarmOBP6 and HarmOBP3 with fatty acids, semiochemicals, triacylglycerols (TAG) and phospholipids

		Binding energy (kcal/mol)		
Ligand	**Ligand**	**OBP6**	**OBP3**	**PBP1[a]**
Fatty acids	1,2-diacylglycerol	−5.1	−4.6	−4.9
	α-linolenic acid	−5.8	−5.7	−7.8
	cis-vaccenic acid	−5.6	−5.3	−6.9
	D-trehalose	−5.2	−5.5	−5.5
	γ-linolenic acid	−6.2	−5.7	−7.5
	Linoleic acid	−5.4	−5.3	−7.3
	L-proline	−4.5	−4.4	−4.5
	Oleic acid	−5.4	−5.5	−7.0
	Palmitic acid	−5.3	−5.1	−6.5
	Palmitoleic acid	−5.5	−5.9	−6.9
	Stearic acid	−5.6	−5.5	−6.7
	trans-vaccenic acid	−5.5	−6.0	−7.1
Semiochemicals	2-phenylacetaldehyde	−5.1	−4.6	−5.9
	2-phenylethanol	−4.9	−4.7	−5.8
	benzaldehyde	−4.8	−4.6	−5.6
	heptanal	−4.2	−3.9	−4.6
	hexadecanal	−5.0	−4.9	−6.5
	hexadecanol	−5.0	−4.8	−6.3
	nonanal	−4.7	−3.9	−5.4
	phenylmethanol	−4.7	−4.8	−5.4
	salicylaldehyde	−5.0	−5.0	−5.2
	tetradecanal	−5.0	−4.5	−6.0
	(*Z*)-7-hexadecenal	−5.2	−5.0	−6.7
	(*Z*)-9-hexadecenal	−5.5	−5.0	−6.6
	(*Z*)-9-tetradecenal	−5.2	−4.8	−6.4
	(*Z*)-11-hexadecenal	−5.4	−4.9	−6.7
	(*Z*)-11-hexadecenol	−5.3	−5.0	−6.4
TAG/phospholipid	triacylglycerol—TAG (52:3)	−13.0	−21.0	−7.5
	triacylglycerol—TAG (52:2)	−18.0	−22.0	50.8
	phosphatidylethanolamine—PE (36:4)	−17.5	−3.8	5.1
	phosphatidylserine—PS (36:2)	−18.0	−19.4	19.1
	phosphatidylinositol—PI (36:3)	−19.9	−21.4	26.0
	phosphatidylglycerol—PG (34:3)	−17.6	−18.7	21.3
	phosphatidylcholines—PC (36:5) (1)[b]	−13.7	−18.2	−0.7
	phosphatidylcholines—PC (36:5) (2)	−14.0	−19.3	−14.8
	phosphatidylcholines—PC (36:5) (3)	−15.3	−19.7	−1.5

[a]HarmPBP1 used as reference target with a reported function in binding sex pheromones.

[b]Numbers in parentheses indicate isomers for phosphatidylcholines as carbon atoms:unsaturations. (1) represents 18:3/18:2; (2) represents 18:2/18:3 and (3) represents 16:0/20:5.

this study have a binding affinity for long-chain fatty acids which is either supplementary to their role in olfaction or represents an entirely new physiological function. There is now compelling evidence that OBPs perform physiological functions beyond olfaction (Pelosi, Iovinella, Zhu, Wang, & Dani, 2018). The sensilla of *Drosophila* maintain a robust response to a wide range of odours even when all abundantly expressed antennal OBP genes are deleted, demonstrating that many OBPs are not essential to the olfactory response (Xiao,

Sun, & Carlson, 2019). Humidity detection (hygrosensation) relies on a single OBP (OBP59a) within *Drosophila* antenna (Sun et al., 2018), contravening the typical model that OBPs exclusively transport hydrophobic odorants to receptors. The diverse array of nonolfactory roles for OBPs in Diptera include bacterial-induced haematopoiesis in tsetse flies (Benoit et al., 2017), the transportation of sex-pheromones in *Helicoverpa* sp. (Sun, Huang, Pelosi, & Wang, 2012) and eggshell formation in the mosquito *Aedes aegypti* (Marinotti et al., 2014). The degree of redundancy in OBP function and the circumstances under which dual or split roles are performed is currently unknown, but tissue-specific functional genomics will undoubtedly begin to uncover the broader range of operation of OBPs.

4 | SUMMARY

It has recently become accepted that the versatility of OBPs is greater than previously thought and this group of proteins represent a highly adaptive set of hydrophobic carriers performing multiple physiological functions beyond their classical role in chemoreception (Pelosi et al., 2018). Our findings on two *Helicoverpa armigera* OBPs are consistent with this view and we propose an additional physiological role in regulating insect flight in a migratory Lepidopteran organism. The affinity of OBPs for long-chain fatty acids (Ishida et al., 2013) lends support to the hypothesis that OBPs act as carriers of hydrophobic free fatty acids produced from upstream lipid metabolism as part of the flight fuel pathway. The OBP homology structure models and binding affinities for a range of substrates described here support this. The precise mechanism(s) of how over-expressed OBPs contribute to flight performance at the biochemical and cellular level needs further study. Coping with the extreme energy demands of sustained migratory flight in insects is just one of several traits that make up the heritable "migratory syndrome" (Roff & Fairbairn, 2007). Investigating the pathways and mechanisms that support such a fascinating feat of endurance is an excellent means to understand animal migration at the genetic level.

ACKNOWLEDGEMENTS

We thank the three anonymous reviewers for their critical comments and suggestions for improvement of the initial draft. The Max Planck Institute in Jena, Germany, and the University of Valencia provided vital *Helicoverpa armigera* insect material. This work was supported by the UK Biotechnology and Biological Sciences Research Council (BBSRC) as part of a Future Leader Fellowship (grant no.: BB/N012011/1) (to C.M.J.) and by Jilin University, China. As part of a Tang Aoqing Chair Professorship (no. TAQ(JZ)-2017[7]-201811) to J.J.Z. L.V.M. is supported by the BBSRC Institute Strategic Programme Tailoring Plant Metabolism (BBS/E/C/000I0420).

AUTHOR CONTRIBUTIONS

C.M.J. and J.J.Z. conceived and designed the study. S.W., M.M., R.A.H., L.V.M. and H.V. performed the experimental work. K.S.L. designed and provided the tethered flight mill equipment. C.M.J., J.J.Z., S.W., A.W., M.M., L.V.M. and H.V. conducted data analysis. J.X. provided advice, academic and financial support for S.W. C.M.J. and J.J.Z. wrote the initial draft of the manuscript. All authors edited and made comments on the final draft.

DATA AVAILABILITY STATEMENT

The candidate gene qPCR, OBP6 tissue-specific expression and lipid quantification ESI-MS data have been archived in Dryad (https://doi.org/10.5061/dryad.dr7sqv9w4). The R script used to analyse the flight mill data is available on request from C.M.J.

ORCID

Rafael A. Homem https://orcid.org/0000-0001-9649-1825
Christopher M. Jones https://orcid.org/0000-0002-6504-6224

REFERENCES

Antunes, D. A., Moll, M., Devaurs, D., Jackson, K. R., Lizée, G., & Kavraki, L. E. (2017). DINC 2.0: A new protein-peptide docking webserver using an incremental approach. *Cancer Research*, *77*(21), E55–E57.

Arrese, E. L., & Soulages, J. L. (2010). Insect fat body: Energy, metabolism, and regulation. *Annual Review of Entomology*, *55*, 207–225. https://doi.org/10.1146/annurev-ento-112408-085356

Bates, D., Machler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48.

Benoit, J. B., Vigneron, A., Broderick, N. A., Wu, Y., Sun, J. S., Carlson, J. R., … Weiss, B. L. (2017). Symbiont-induced odorant binding proteins mediate insect host hematopoiesis. *eLife*, *6*, e19535. https://doi.org/10.7554/eLife.19535

Bischof, J., Maeda, R. K., Hediger, M., Karch, F., & Basler, K. (2007). An optimized transgenesis system for Drosophila using germ-line-specific phi C31 integrases. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(9), 3312–3317.

Brand, A. H., & Perrimon, N. (1993). Targeted gene-expression as a means of altering cell fates and generating dominant phenotypes. *Development*, *118*(2), 401–415.

Brand, P., Robertson, H. M., Lin, W., Pothula, R., Klingeman, W. E., Jurat-Fuentes, J. L., & Johnson, B. R. (2016). The origin of the odorant receptor gene family in insects. *Elife*, *7*, e38340.

Bruce, T. J. A., & Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects—Finding the right mix. *Phytochemistry*, *72*(13), 1605–1611. https://doi.org/10.1016/j.phytochem.2011.04.011

Chapman, J. W., Nesbit, R. L., Burgin, L. E., Reynolds, D. R., Smith, A. D., Middleton, D. R., & Hill, J. K. (2010). Flight orientation behaviors promote optimal migration trajectories in high-flying insects. *Science*, *327*(5966), 682–685. https://doi.org/10.1126/science.1182990

Chertemps, T., François, A., Durand, N., Rosell, G., Dekker, T., Lucas, P., & Maïbèche-Coisne, M. (2012). A carboxylesterase, Esterase-6, modulates sensory physiological and behavioral response dynamics to pheromone in Drosophila. *Bmc Biology*, *10*, 56. https://doi.org/10.1186/1741-7007-10-56

Delignette-Muller, M. L., & Dutang, C. (2015). fitdistrplus: An R Package for Fitting Distributions. *Journal of Statistical Software*, *64*(4), 1–34.

Devaiah, S. P., Roth, M. R., Baughman, E., Li, M., Tamura, P., Jeannotte, R., … Wang, X. (2006). Quantitative profiling of polar glycerolipid species from organs of wild-type Arabidopsis and a phospholipase D alpha 1 knockout mutant. *Phytochemistry*, *67*(17), 1907–1924.

Dhanik, A., McMurray, J. S., & Kavraki, L. E. (2013). DINC: A new AutoDock-based protocol for docking large ligands. *BMC Structural Biology*, *13*, S11. https://doi.org/10.1186/1472-6807-13-S1-S11

Dingle, H. (2014). *Migration: The biology of life on the move*, 2nd ed. Oxford, UK: Oxford University Press.

Dong, K., Sun, L., Liu, J. T., Gu, S. H., Zhou, J. J., Yang, R. N., … & Zhang, Y. J. (2017). RNAi-induced electrophysiological and behavioral changes reveal two pheromone binding proteins of Helicoverpa armigera involved in the perception of the main sex pheromone component Z11–16:Ald. *Journal of Chemical Ecology*, *43*(2), 207–214. https://doi.org/10.1007/s10886-016-0816-6

Dundas, J., Ouyang, Z., Tseng, J., Binkowski, A., Turpaz, Y., & Liang, J. (2006). CASTp: Computed atlas of surface topography of proteins with structural and topographical mapping of functionally annotated residues. *Nucleic Acids Research*, *34*, W116–W118. https://doi.org/10.1093/nar/gkl282

Fitt, G. P. (1989). The ecology of Heliothis species in relation to agroecosystems. *Annual Review of Entomology*, *34*, 17–52. https://doi.org/10.1146/annurev.en.34.010189.000313

Getahun, M. N., Thoma, M., Lavista-Llanos, S., Keesey, I., Fandino, R. A., Knaden, M., … Hansson, B. S. (2016). Intracellular regulation of the insect chemoreceptor complex impacts odour localization in flying insects. *Journal of Experimental Biology*, *219*(21), 3428–3438. https://doi.org/10.1242/jeb.143396

Gomez-Diaz, C., Reina, J. H., Cambillau, C., & Benton, R. (2013). Ligands for pheromone-sensing neurons are not conformationally activated odorant binding proteins. *Plos Biology*, *11*(4), e1001546. https://doi.org/10.1371/journal.pbio.1001546

Gong, D. P., Zhang, H. J., Zhao, P., Xia, Q. Y., & Xiang, Z. H. (2009). The odorant binding protein gene family from the genome of silkworm, *Bombyx mori*. *BMC Genomics*, *10*(1), 332. https://doi.org/10.1186/1471-2164-10-332

Graham, L. A., Tang, W., Baust, J. G., Liou, Y. C., Reid, T. S., & Davies, P. L. (2001). Characterization and cloning of a Tenebrio molitor hemolymph protein with sequence similarity to insect odorant-binding proteins. *Insect Biochemistry and Molecular Biology*, *31*(6–7), 691–702. https://doi.org/10.1016/S0965-1748(00)00177-6

Grosse-Wilde, E., Svatos, A., & Krieger, J. (2006). A pheromone-binding protein mediates the bombykol-induced activation of a pheromone receptor in vitro. *Chemical Senses*, *31*(6), 547–555. https://doi.org/10.1093/chemse/bjj059

Gu, H. N., & Danthanarayana, W. (1992). Quantitative genetic analysis of dispersal in *Epiphyas postvittana*. 1. Genetic variation in flight capacity. *Heredity*, *68*, 53–60.

Gu, S.-H., Sun, L., Yang, R.-N., Wu, K.-M., Guo, Y.-Y., Li, X.-C., … Zhang, Y.-J. (2014). Molecular characterization and differential expression of olfactory genes in the antennae of the black cutworm moth agrotis ipsilon. *PLoS One*, *9*(8), e103420. https://doi.org/10.1371/journal.pone.0103420

Guo, W., Wang, X., Ma, Z., Xue, L., Han, J., Yu, D., & Kang, L. (2011). CSP and takeout genes modulate the switch between attraction and repulsion during behavioral phase change in the migratory locust. *PLoS Genetics*, *7*(2), e1001291. https://doi.org/10.1371/journal.pgen.1001291

Hansson, B. S., & Stensmyr, M. C. (2011). Evolution of insect olfaction. *Neuron*, *72*(5), 698–711. https://doi.org/10.1016/j.neuron.2011.11.003

Ishida, Y., Ishibashi, J., & Leal, W. S. (2013). Fatty acid solubilizer from the oral disk of the blowfly. *PLoS One*, *8*(1), e51779. https://doi.org/10.1371/journal.pone.0051779

Jones, C. M., Papanicolaou, A., Mironidis, G. K., Vontas, J., Yang, Y., Lim, K. S., … Chapman, J. W. (2015). Genomewide transcriptional signatures of migratory flight activity in a globally invasive insect pest. *Molecular Ecology*, *24*(19), 4901–4911. https://doi.org/10.1111/mec.13362

Jones, C. M., Parry, H., Tay, W. T., Reynolds, D. R., & Chapman, J. W. (2019). Movement ecology of pest Helicoverpa: Implications for ongoing spread. *Annual Review of Entomology*, *64*, 277–295.

Huang, J., Rauscher, S., Nawrocki, G., Ran, T., Feig, M., De Groot, B. L., … & MacKerell, A. D. (2016). CHARMM36m: An improved force field for folded and intrinsically disordered proteins. *Nature Methods*, *14*(1), 71–73. https://doi.org/10.1038/nmeth.4067

Krank, J., Murphy, R. C., Barkley, R. M., Duchoslav, E., & McAnoy, A. (2007). Qualitative analysis and quantitative assessment of changes in neutral glycerol lipid molecular species within cells. *Methods in Enzymology*, *432*, 1–20.

Larter, N. K., Sun, J. S., & Carlson, J. R. (2016). Organization and function of Drosophila odorant binding proteins. *Elife*, *5*, e20242. https://doi.org/10.7554/eLife.20242

Lartigue, A., Campanacci, V., Roussel, A., Larsson, A. M., Jones, T. A., Tegoni, M., & Cambillau, C. (2002). X-ray structure and ligand binding study of a moth chemosensory protein. *Journal of Biological Chemistry*, *277*(35), 32094–32098. https://doi.org/10.1074/jbc.M204371200

Laskowski, R. A., Macarthur, M. W., Moss, D. S., & Thornton, J. M. (1993). PROCHECK—A programme to check the stereochemical quality of protein structures. *Journal of Applied Crystallography*, *26*, 283–291.

Leal, W. S. (2013). Odorant reception in insects: Roles of receptors, binding proteins, and degrading enzymes. *Annual Review of Entomology*, *58*, 373–391. https://doi.org/10.1146/annurev-ento-120811-153635

Lenth, R. V. (2016). Least-squares means: The R package lsmeans. *Journal of Statistical Software*, *69*(1), 1–33.

Li, Z.-Q., Zhang, S., Luo, J.-Y., Wang, C.-Y., Lv, L.-M., Dong, S.-L., & Cui, J.-J. (2015). Transcriptome comparison of the sex pheromone glands from two sibling Helicoverpa species with opposite sex pheromone components. *Scientific Reports*, *5*, 9324. https://doi.org/10.1038/srep09324

Liedvogel, M., Akesson, S., & Bensch, S. (2011). The genetics of migration on the move. *Trends in Ecology & Evolution*, *26*(11), 561–569. https://doi.org/10.1016/j.tree.2011.07.009

Marinotti, O., Ngo, T., Kojin, B. B., Chou, S. P., Nguyen, B., Juhn, J., … James, A. A. (2014). Integrated proteomic and transcriptomic analysis of the *Aedes aegypti* eggshell. *BMC Developmental Biology*, *14*, 15.

McCormick, A. C., Grosse-Wilde, E., Wheeler, D., Mescher, M. C., Hansson, B. S., & De Moraes, C. M. (2017). Comparing the expression of olfaction-related genes in gypsy moth (*Lymantria dispar*) adult females and larvae from one flightless and two flight-capable populations. *Frontiers in Ecology and Evolution*, *5*, 115. https://doi.org/10.3389/fevo.2017.00115

Merlin, C., &Liedvogel, M. (2019). The genetics and epigenetics of animal migration and orientation: Birds, butterflies and beyond. *Journal of Experimental Biology*, *222*, jeb191890. https://doi.org/10.1242/jeb.191890

Mescher, M. C., & De Moraes, C. M. (2015). Role of plant sensory perception in plant–animal interactions. *Journal of Experimental Botany*, *66*(2), 425–433. https://doi.org/10.1093/jxb/eru414

Miller, D. F. B., Holtzman, S. L., & Kaufman, T. C. (2002). Customized microinjection glass capillary needles for P-element transformations in *Drosophila melanogaster*. *BioTechniques*, *33*(2), 366–375.

Minter, M., Pearson, A., Lim, K. S., Wilson, K., Chapman, J. W., & Jones, C. M. (2018). The tethered flight technique as a tool for studying life-history strategies associated with migration in insects. *Ecological Entomology*, *43*(4), 397–411. https://doi.org/10.1111/een.12521

Missbach, C., Vogel, H., Hansson, B. S., & Grosse-Wilde, E. (2015). Identification of odorant binding proteins and chemosensory proteins in antennal transcriptomes of the jumping bristletail lepismachilis y-signata and the firebrat thermobia domestica: Evidence for an independent OBP-OR Origin. *Chemical Senses*, *40*(9), 615–626.

Olzmann, J. A., & Carvalho, P. (2019). Dynamics and functions of lipid droplets. *Nature Reviews Molecular Cell Biology*, *20*(3), 137–155. https://doi.org/10.1038/s41580-018-0085-z

Pelosi, P., Iovinella, I., Felicioli, A., & Dani, F. R. (2014). Soluble proteins of chemical communication: An overview across arthropods. *Frontiers in Physiology*, *5*, 320. https://doi.org/10.3389/fphys.2014.00320

Pelosi, P., Iovinella, I., Zhu, J., Wang, G. R., & Dani, F. R. (2018). Beyond chemoreception: Diverse tasks of soluble olfactory proteins in insects. *Biological Reviews*, *93*(1), 184–200. https://doi.org/10.1111/brv.12339

Pelosi, P., Zhou, J. J., Ban, L. P., & Calvello, M. (2006). Soluble proteins in insect chemical communication. *Cellular and Molecular Life Sciences*, *63*(14), 1658–1676. https://doi.org/10.1007/s00018-005-5607-0

Reppert, S. M., Guerra, P. A., & Merlin, C. (2016). Neurobiology of monarch butterfly migration. *Annual Review of Entomology*, *61*(1), 25–42. https://doi.org/10.1146/annurev-ento-010814-020855

Rio, R. V. M., Attardo, G. M., & Weiss, B. L. (2016). Grandeur alliances: Symbiont metabolic integration and obligate arthropod hematophagy. *Trends in Parasitology*, *32*(9), 739–749.

Roff, D. A., & Fairbairn, D. J. (2007). The evolution and genetics of migration in insects. *BioScience*, *57*(2), 155–164. https://doi.org/10.1641/B570210

Ruiz-Lopez, N., Haslam, R. P., Napier, J. A., & Sayanova, O. (2014). Successful high-level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids in a transgenic oilseed crop. *Plant Journal*, *77*(2), 198–208. https://doi.org/10.1111/tpj.12378

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C-T method. *Nature Protocols*, *3*(6), 1101–1108. https://doi.org/10.1038/nprot.2008.73

Seroude, L., Brummel, T., Kapahi, P., & Benzer, S. (2002). Spatio-temporal analysis of gene expression during aging in Drosophila melanogaster. *Aging Cell*, *1*(1), 47–56. https://doi.org/10.1046/j.1474-9728.2002.00007.x

Steinhauser, M. L., Olenchock, B. A., O'Keefe, J., Lun, M., Pierce, K. A., Lee, H., … Fazeli, P. K. (2018). The circulating metabolome of human starvation. *JCI Insight*, *3*(16), e121434. https://doi.org/10.1172/jci.insight.121434

Sun, J. S., Larter, N. K., Chahda, J. S., Rioux, D., Gumaste, A., & Carlson, J. R. (2018). Humidity response depends on the small soluble protein Obp59a in Drosophila. *eLife*, *7*, e39249. https://doi.org/10.7554/eLife.39249

Sun, Y.-L., Huang, L.-Q., Pelosi, P., & Wang, C.-Z. (2012). Expression in antennae and reproductive organs suggests a dual role of an odorant-binding protein in two sibling *Helicoverpa* species. *PLoS One*, *7*(1), e30040.

Tegoni, M., Campanacci, V., & Cambillau, C. (2004). Structural aspects of sexual attraction and chemical communlication in insects. *Trends in Biochemical Sciences*, *29*(5), 257–264.

Trott, O., & Olson, A. J. (2010). Software news and update AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, *31*(2), 455–461.

Tsuchihara, K., Fujikawa, K., Ishiguro, M., Yamada, T., Tada, C., Ozaki, K., & Ozaki, M. (2005). An odorant-binding protein facilitates odorant transfer from air to hydrophilic surroundings in the blowfly. *Chemical Senses*, *30*(7), 559–564. https://doi.org/10.1093/chemse/bji049

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3-new capabilities and interfaces. *Nucleic Acids Research*, *40*(15), e115. https://doi.org/10.1093/nar/gks596

Usher, S., Han, L., Haslam, R. P., Michaelson, L. V., Sturtevant, D., Aziz, M., … Napier, J. A. (2017). Tailoring seed oil composition in the real world: Optimising omega-3 long chain polyunsaturated fatty acid accumulation in transgenic Camelina sativa. *Scientific Reports*, *7*, 6570. https://doi.org/10.1038/s41598-017-06838-0

Van der Horst, D. J., & Ryan, R. O. (2012). *Lipid transport* (pp. 317–345). New York: Academic Press.

van der Veen, J. N., Kennelly, J. P., Wan, S., Vance, J. E., Vance, D. E., & Jacobs, R. L. (2017). The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. *Biochimica Et Biophysica Acta (BBA) - Biomembranes*, *1859*(9), 1558–1572. https://doi.org/10.1016/j.bbamem.2017.04.006

Vieira, F. G., & Rozas, J. (2011). Comparative genomics of the odorant-binding and chemosensory protein gene families across the Arthropoda: Origin and evolutionary history of the chemosensory system. *Genome Biology and Evolution*, *3*, 476–490. https://doi.org/10.1093/gbe/evr033

Wang, Z. J., Dong, Y. C., Desneux, N., & Niu, C. Y. (2013). RNAi silencing of the HaHMG-CoA reductase gene inhibits oviposition in the *Helicoverpa armigera* cotton bollworm. *PLoS One*, *8*(7), e67732. https://doi.org/10.1371/journal.pone.0067732

Xiao, S., Sun, J. S., & Carlson, J. R. (2019). Robust olfactory responses in the absence of odorant binding proteins. *eLife*, *8*, e51040. https://doi.org/10.7554/eLife.51040

Yan, S., Ni, H., Li, H., Zhang, J., Liu, X., & Zhang, Q. (2013). Molecular cloning, characterization, and mRNA expression of two cryptochrome genes in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology*, *106*(1), 450–462.

Ye, Z. F., Liu, X. L., Han, Q., Liao, H., Dong, X. T., Zhu, G. H., & Dong, S. L. (2017). Functional characterization of PBP1 gene in Helicoverpa armigera (Lepidoptera: Noctuidae) by using the CRISPR/Cas9 system. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-08769-2

Zhan, S., Zhang, W., Niitepõld, K., Hsu, J., Haeger, J. F., Zalucki, M. P., … Kronforst, M. R. (2014). The genetics of monarch butterfly migration and warning colouration. *Nature*, *514*(7522), 317–321. https://doi.org/10.1038/nature13812

Zhou, J. J. (2010). Odorant-binding proteins in insects. In G. Litwack (Ed.), *Vitamins and hormones: Pheromones* (vol. *83*, pp. 241–272). Academic Press.

Zhou, J. J., He, X. L., Pickett, J. A., & Field, L. M. (2008). Identification of odorant-binding proteins of the yellow fever mosquito Aedes aegypti: Genome annotation and comparative analyses. *Insect Molecular Biology*, *17*(2), 147–163. https://doi.org/10.1111/j.1365-2583.2007.00789.x

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Wang S, Minter M, Homem RA, et al. Odorant binding proteins promote flight activity in the migratory insect, *Helicoverpa armigera*. *Mol Ecol*. 2020;00:1–14. https://doi.org/10.1111/mec.15556