THE INTERACTION BETWEEN MIGRATION AND DISEASE IN THE FALL ARMYWORM, *SPODOPTERA FRUGIPERDA*

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

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ABSTRACT

Every year billions of insects undertake long-distance seasonal migrations, moving hundreds of tonnes of biomass across the globe and providing key ecological services. Yet we know very little about the complex migratory movements of these tiny animal migrants and less still about what causes their populations to fluctuate in space and time. Understanding the reason for these population level changes is important, especially for insect species that are agricultural pests and disease vectors. One possible driver of large scale population dynamics in migratory insect species is disease. Migration is a stressful and energetically-costly behaviour. Fighting off, or living with, infections is also costly. In migratory animals that have been exposed to disease this may lead to potential trade-offs between investment in migration and investment in the resistance and tolerance mechanisms associated with infection. Using a combination of rotational flight mills, bioassays and molecular techniques, this thesis uses the fall armyworm, *Spodoptera frugiperda*, and its associated baculovirus, *S. frugiperda* multiple nucleopolyhedrovirus (*Sf*MNPV), as a model system to describe the trade-off between migratory effort and disease susceptibility, and how this affects disease dynamics at a geographic scale. After a general introduction to the topic (Chapter 1), Chapter 2 uses an inter-species analysis to describe the insect flight patterns associated with migratory behaviour in three species of migratory noctuid moth, linking these with previous work on the upregulation of genes associated with the migratory syndrome and providing evidence of sex-biased dispersal in the fall armyworm. Chapter 3 builds on these results by quantifying the impact of infection on migratory flight behaviour, and provides the first evidence of Bateman's principle in insect migrants by demonstrating that males and females exhibit different developmental and physiological responses to infection, and adopt different flight strategies following virus exposure. To understand how this affects susceptibility, Chapter 4 quantifies the effect of flight effort on resistance to infection, showing that prolonged bouts of flight results in an increase in disease loads but only in populations with low levels of background infection. This provides evidence that the trade-off between flight effort and resistance is context dependent and possibly phenotypically plastic. Finally, Chapter 5 contextualises these laboratory results by investigating fluctuations in disease load across the United States of America. Findings from this study show that the host-pathogen system is relatively stable over large geographic distances and time periods of up to two years. Where variation does occur, there is evidence for 'escape' from infection but that this is often associated with the cost of reduced resource availability in males. Overall the work demonstrates key physiological and behavioural adaptations that enable insects to engage in long-distance migration when faced with competing costs of flight and disease resistance.

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CHAPTER 1.

GENERAL INTRODUCTION

Migratory disease ecology is a field that has only begun to emerge in the last five to ten years (Altizer et al., 2011). The theoretical aspects are still evolving and more effort is required to combine the theoretical and experimental knowledge of animal migration on the one hand and disease ecology on the other. Advances have been made however, and this chapter attempts to describe what has been developed over the last five years and to frame it within the context of insect migration, and what we can learn about the relationship between migration and disease from baculovirus dynamics in noctuid moths.

To achieve this, section 1.1 describes different animal movement paradigms, defining migration and how this relates to hosts and pathogens. With this definition in place, section 1.2 gives an overview of insect migration, particularly the migratory syndrome (section 1.2.2) and the drivers of migration (section 1.2.1). As a brief introduction, and to provide contextual background to the methods used in this thesis, section 1.2.3 describes the different ways in which insect migration is studied, both in the field and in the laboratory.

Following on from this, section 1.3 looks at baculoviruses, and how our knowledge of their disease ecology is relevant to insect migration. There is a general introduction to these pathogens, following which three key aspects (disease transmission, population dynamics and baculovirus diversity) are described. This is followed by a description of the primary host-pathogen system used in this thesis: the fall armyworm *Spodoptera* frugiperda (J.E. Smith) and its baculovirus *S. frugiperda* multiple nucleopolyhedrovirus (*Sf*MNPV) (section1.4).

Finally, section 1.5 describes what we currently know of the relationships between migration and disease, giving an overview of the different theories to date and what level of support there is for these in the literature. This is followed by the theoretical approach used in this thesis and a brief overview of each of the following chapters (section 1.5.7).

1.1 MOVEMENT PARADIGMS AND THEIR RELATIONSHIP TO HOST-PATHOGEN INTERACTIONS

Movement is a key behavioural life-history trait, essential to the establishment and reproductive success of individuals and species alike (Dingle, 2014). It is a trait upon which there is ample possibility for selection to act (Ronce, 2007, Shaw and Couzin, 2013) and it is directly linked to ecosystem functioning and biodiversity (Bauer and Hoye, 2014), affecting the dynamics and stability of meta-communities and driving key landscape scale processes such as nutrient cycling (Landry and Parrott, 2016). As such, an understanding of movement ecology is essential for everything from conservation to vector control and agriculture. Yet to date, the definitions and distinctions that allow us to explain the wide variety of movement behaviours are still debated (Nathan et al., 2008, Clobert et al., 2009, Dingle, 2014). The terms migration and dispersal are often used interchangeably, particularly in relation to insects.

Unlike mammals and birds, where annual movement between breeding and non-breeding habitats is common, individual round-trip migration in insects is rarely documented (Holland et al., 2006). Instead, long-distance movement in insects is generally multi-generational; a succession of one-way movements undertaken by multiple consecutive generations through a series of breeding grounds, so that over a migratory season the population as a whole may follow a return trajectory but no single individual will complete the journey (Drake and Gatehouse, 1995).

Within the field of dispersal ecology, the definition of migration focuses on the drivers of animal movement at a population scale, and how this relates to variation in phenotypes and settlement behaviour (Clobert et al., 2009). By this definition, long-distance insect movement would be classed as 'dispersive' rather than 'migratory' because every generation results in a relocation of the natal/ breeding site.

A more commonly accepted framework in entomology (and that used in this thesis) is the 'taxonomy of movement' described by Dingle (2014). This framework defines different movement categories at an individual level: based on the underlying behavioural and physiological traits *while moving*. It also broadly divides movement behaviour into two different categories: movements which are under control of the organism, and those which are not.

Insect hosts: movement which is under the control of the organism

In terms of disease dynamics, the movement behaviours which are under control of the organism are usually those of the host (Dingle, 2014): station keeping (movements which keep an animal inside its home range, and which may contain sub-behaviours such as foraging or territorial

behaviour); ranging (movement over a habitat which ceases when a suitable home range is located) and migration, where the standard working definition used in entomology is that of J. S. Kennedy:

> "Migratory behaviour is the persistent and straightened out movement effected by the animals 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)

However, it is acknowledged that not all migrants show all characteristics of migration (Dingle and Drake, 2007) and that movement behaviours may grade into one another (Fryxell et al., 2008), especially when trying to apply a definition across a broad range of taxa with very different strategies. As such, the definition remains a broad working term and it is important to bear in mind that movement behaviour exists on a continuum upon which selection continues to act (Southwood, 1962).

Pathogen movement behaviour: movement which is not under the control of the organism?

In the context of movement behaviour as defined by Dingle (2014), those which are not under the control of the organism are accidental displacement ("organism does not initiate movement. Movement stops when organism leaves transporting vehicle") and assisted migration or assisted transport ("accidental or anthropogenic movement, for example conservation introductions").

How these descriptions, or indeed the definition of migration above, relates to pathogens is one of the theoretical links that needs to be considered further in disease ecology, but is worth investigating because the spatio-temporal co-occurrence of host and pathogen is an essential part of disease transmission (Bauer et al., 2016). This is complicated enough for organisms such as bacteria and protozoa, which have low levels of self-powered motility but are at least included in the tree of life. For viruses, where there is some debate as to whether or not they are living organisms (Moreira and Lopez-Garcia, 2009), this is likely to be trickier still.

One option perhaps is to consider pathogen dispersal in terms of transmission mechanism and strategy, which may be either active or passive. For example, accidental displacement implies that this form of movement is not strictly 'behavioural', but rather forces movement which is beyond the organism's control. For pathogens that are able to survive outside the host for prolonged periods, this might occur as a result of chance events such as rainfall (D'Amico and Elkinton, 1995) or habitat disturbance (Crawford and Kalmakoff, 1977). Importantly, in such instances we could assume that the distance the organism is carried would be random and this would exert selection pressures on the pathogen that are likely to affect how it survives outside the host.

By contrast, for pathogens which successfully infect and are carried by a host, the distance moved would not be random but rather very closely tied to that of the host. In such instances, transmission is likely to be an active process on the part of the pathogen and may even fit within the concept of 'active embarkation on a vehicle' in Kennedy's definition of migration. It is also worth noting that a pathogen may directly or indirectly influence the host's movement behaviour. For example, in a review of the effects of parasites in biting flies, a wide variety of behavioural changes were documented, from decreases in activity to hyper-activity, flight muscle damage and no effect at all (Moore, 1993). Another particularly well documented example of the effect of infection on host movement is rabies. Lyssaviruses, the causative agents, are very fragile and do not persist in the environment outside their host, yet in their canine hosts they been shown to lead to an increase in aggression that is also associated with movement behaviour that pushes them outside of their normal home range (Rupprecht et al., 2002).

These considerations may be relevant in relation to the vertical and horizontal transmission strategies documented in baculoviruses (sections 1.3.1).

1.2 INSECT MIGRATION

Insects form the most speciose and abundant group of territorial animal migrants, and as vectors of zoonotic diseases, major pests of agricultural systems and providers of key ecosystems services, the consequences of these movements can have major implications, making this group the most economically important group of terrestrial migrants (Chapman et al., 2015b, Hu et al., 2016). Yet annual migration routes of migratory insects remain poorly characterised (Drake and Gatehouse, 1995) (section1.2) and we know far less about the costs and benefits of migration in insects than we do in birds and mammals (section 1.2.1).

While migration may be obligatory in a habitat that can only support a single generation, most insects are facultative migrants, migrating in response to a variety of environmental cues. This adaptation allows individuals to exploit a variety of ephemeral habitats over space and time, and reduce direct competition by occupying alternative ecological niches (Southwood, 1962). As such, insect migration generally has a semi-nomadic quality, where movement between habitats only occurs when conditions are favourable (Dingle and Drake, 2007). Where a habitat can support multiple generations, it also can't be assumed that all individuals in a source population will migrate. The result of this is that migratory routes, patterns and populations are not regular, and can be difficult to define for both biological and technical reasons (section 1.2.3). The sudden arrival of migrants in the summer months or, in arid regions, in response to rainfall, allows us to study early migratory movements with reasonable levels of certainty, but the challenge of differentiating returning migrants from resident populations means the study of return migration is less well documented. This initially lead to the development of the "Pied Piper" hypothesis which postulated that spring movements to high latitudes were doomed (Dingle, 1982) (Dantart et al., 2009), but in recent years developments in radar technology and citizen science has led to return migration being well described in several species, particularly the Monarch butterfly *Danaus plexippus* (Flockhart et al., 2013) and the silver Y moth, *Autographa gamma* (Chapman et al., 2012). In this case, the "Pied Piper" theory looks less plausible although the details of these return migrations are still largely unknown.

1.2.1 EVOLUTIONARY DRIVERS, ECOLOGICAL STRATEGIES AND CONSEQUENCES FOR POPULATION DYNAMICS

The extent to which migration occurs depends on a wide variety of site-specific factors; the fundamental drivers of migration. In recent years, a wider variety of influences has begun to be considered (Chapman et al., 2015b), including disease (section 1.5) (Altizer et al., 2011). The primary driver however is still assumed to be resource availability, which is closely related to weather and climate (Drake and Gatehouse, 1995), particularly where changes result in

temporary habitat availability. Indeed, the more ephemeral the habitat, the higher the level of movement, or at least, in planthoppers and other hemipterans (Denno et al., 1996).

Rainfall and temperature in particular are considered to be fundamental selection pressures and migratory events are generally considered to either track seasonal changes in plant availability (Drake and Reynolds, 2012) (Chapman et al., 2012) or, in arid and semi-arid tropical and sub-tropical zones, in response to rainfall patterns, in which case they are often less predictable (Drake and Gatehouse, 1995, Rose et al., 2000). Meteorological conditions are particularly known to affect flight strategies, with insect migrants loosely classified into two different groups: day flying and night flying (Chapman et al., 2015b). Day flying insect migrants are often large, ectothermic species like butterflies, which typically migrate close to the ground in their flight boundary layer (FBL) (Taylor, 1974, Srygley and Dudley, 2008), the lowest few metres of the atmosphere within which each species' self-powered airspeed exceeds the mean wind speed. However, recent studies have suggested that insect migrations can also occur at much higher altitudes during the day (Chapman et al., 2010, Hu et al., 2016). The fact that the self-powered flight of these FBL migrants exceeds the wind speed gives them a high level of control over their migratory direction, although contending with the turbulence that results from thermal convection is generally considered to be strenuous. Day flying insects are also considered to be more exposed to predators (Anderson, 2009). In large species, such as macro-moths, nocturnal migration is far more common with insects making use of high speed, low-level jets that form as inversion layers several hundred meters above the ground during the night (Chapman et al., 2010, Reynolds et al., 2008). Among other things, this has the advantage of faster displacements and avoidance of day-flying predators but at the cost of having less directional control, although larger insects are able to choose winds best suited to their migration and are able to maintain active downwind headings despite changes in wind direction with altitude and/ or time (Chapman et al., 2011b, Chapman et al., 2015a, Chapman et al., 2008). Smaller insects, such as planthoppers, appear to have far less control (Riley et al., 1991) and the variety of 'preferred inherited directions' is more variable across species, suggesting that these insects may utilise a different migratory strategy whereby random headings could increase population divergence (Chapman et al., 2015b).

In terms of the costs and benefits of migration, most research in insects has focused on short-range, dispersive movement (Bonte et al., 2012) and there is far less information available for insects than there is for other taxa. In particular, very little is known about the costs of migration in insects. Typically, these are assumed to be reduced survival and/or fecundity, usually as a result of the cost of flight 'machinery' and the energy required to power insect flight, which is the most energetically expensive form of animal locomotion (Bonte et al., 2012, Reinhold, 1999). However,

recent work has begun to somewhat question the simplicity of this perception. Across a wide range of taxa, migrants have been found to invest more in reproduction than non-migrants (Stevens et al., 2014) and where this has been studied in the silver Y moth, a net increase in population size has been observed over the migratory season (Chapman et al., 2012). Mortality in the same species was also found to be surprisingly low, with approximately 80% of emigrants reaching their destination (Chapman et al., 2012). There is some evidence to suggest that migration can lower the risk of predation, parasitism and disease (Altizer et al., 2011).

1.2.2 THE MIGRATION SYNDROME

Across a wide range of species from many different taxa, a suite of behavioural, physiological, biochemical and morphological adaptations is associated with migration. These characteristics are absent in non-migratory species and include traits such as macroptery – the presence of functional wings (Denno et al., 1996), the accumulation of lipids for flight (Dudley and Srygley, 2008), demographic changes such as increases in development rates (Rose et al., 2000) and alterations in behaviour (Kennedy, 1951). In combination, this suite of co-adapted traits is referred to as a migration syndrome (Dingle and Drake, 2007, Roff and Fairbairn, 2007) or in some instances as dispersal syndromes (Clobert et al., 2009, Stevens et al., 2014).

One trait that has been studied in particular detail is the relationship between migration and reproduction, or what is referred to as the 'oogenesis-flight syndrome' (Johnson, 1969). In many migratory species, this is a decrease in migratory behaviour with the onset of reproductive development, such that migration is usually confined to what is called the pre-reproductive period (PRP). It is assumed that this occurs because it makes more resources available for migration prior to reproduction, however not all species exhibit this behaviour and it is somewhat debated in the literature (e.g. (Sappington and Showers, 1992).

This migratory syndrome is coordinated by a genetic complex (Gatehouse et al., 1989, Jones et al., 2015, Zhan et al., 2014). Most of the traits are polygenetic (i.e. they depend on genes at several different loci) and show continuous variation (Gatehouse et al., 1989, Gatehouse and Zhang, 1995). Thus, there is often an unbroken range of migratory phenotypes in the population. It is this diversity that selection acts upon, driving evolutionary change along particular trajectories (Gatehouse and Zhang, 1995). Many of the traits are also genetically correlated and subject to genotype x environment interactions (Dingle, 2014), which are modulated by external cues such as food availability (Roff and Gélinas, 2003) (King et al., 2011) and population densities (Woodrow et al., 1987). Recent work has begun to elucidate some of the candidate genes involved in this syndrome and identified genes from a wide range of physiological adaptations, from the mobilisation of lipids to the regulation of hormones and the development of flight

muscles (Jones et al., 2015). In combination with the wide range of migratory phenotypes observed in field populations, this complex set of pathways suggests a remarkable flexibility within migratory populations to respond to the highly variable climatic conditions and the resulting vagaries of spatio-temporal unpredictability in habitat over multiple migratory seasons.

1.2.3 METHODS FOR STUDYING INSECT MOVEMENT

Tracking animals as they disperse has always been a challenge. In birds and larger animals, the use of radio telemetry, GPS tagging and satellite tracking are common practice and allow the paths of multiple individuals to be mapped over very large spatial scales. In the insects, the dispersal distance can match those of much larger mammals but their small size and lack of return to nest sites (eusocial insects are something of an exception) creates a unique set of problems. In particular, the batteries used to power many devices are simply too big to be carried by insects, and as a result the study of insect movement to some extent lags behind that of larger animals (Chapman et al., 2015b). This is particularly so in relation to mapping population trajectories through space and time, a major objective of conservation, vector control and pest management (Dingle and Drake, 2007, Drake and Gatehouse, 1995).

The small size of insects however can also be an advantage, as they are far more amenable to laboratory and large scale population studies. Tethered-flight has given us insights into insect flight behaviour that are simply not possible with larger animals (see chapter 2). Within the laboratory, these studies often manipulate environmental conditions and tend to take one of two forms: tethered or free-flying flight. Free-flying studies make use of air treadmills or vertical flight chambers (Blackmer et al., 2004), but tethered flight studies are more common and have been used to assess flight behaviour in a wide range of insect groups, from flies and true bugs to butterflies and beetles (Appendix 1). These studies take a variety of different forms and can be used to study a wide range of different behaviours. Flight simulators are commonly used to investigate orientation strategies, for example in actively migrating butterflies (Nesbit et al., 2009, Mouritsen and Frost, 2002), while flight periodicity is commonly studied using tethered flight mills to investigate take-off and landing times, and even oviposition rates (Colvin and Gatehouse, 1993). Finally, the technique used in this thesis is rotational flight mills (Chambers et al., 1976). Detailed fully in Chapter 2, these flight mills enable the measurement of continuous flight bouts over prolonged periods of time, enabling detailed insights into how flight parameters such as duration, speed and periodicity vary under experimentally manipulated conditions. However, given the artificial nature of the system, flight mills have rarely been used to investigate flight behaviour (see chapter 2).

At a field scale, there are multiple different technologies that allow us to make observations of insect flight patterns. Caged experiments have been used to give insights into flight propensity and habitat boundaries (Norberg et al., 2002) while harmonic radar (a small, light-weight radio transmitter attached to the insect's upper thorax which retransmits signal from a specially designed radar) has enabled novel studies of foraging and local flight behaviour (Woodgate et al., 2016).

At a larger spatial scale, national trapping networks such as those used in Chapter 5 have proved invaluable, providing useful information to conservationists and agronomists alike (Taylor, 1986, Fox et al., 2013). Multisite mark-release-recapture studies (MRR) have given us some insight into dispersal abilities and daily displacement rates, although this is often liable to inherent environmental and study biases (Franzen and Nilsson, 2007). Scanning and vertical looking radar, particularly when combined with trajectory analysis and aerial netting, have also lead to insights into the altitude, orientation, direction and speed of migratory insects, answering fundamental questions relating to insect migration (Chapman et al., 2012, Chapman et al., 2010).

Molecular studies have also proved their value in helping researchers understand gene-flow, and hence movement, between populations and this has been of particular value in the fall armyworm (Nagoshi et al., 2012). More unusual techniques include light-weight radio transmitters which can be attached to larger insects and are tracked by ground-based teams and light aircraft (Wikelski et al., 2006), and in recent years, citizen science data has also been shown to be valuable (Flockhart et al., 2013). Stable isotope analysis, which is common in studies of migratory birds, has proven useful but is still in its infancy in insects (Rubenstein and Hobson, 2004, Nagoshi et al., 2007a).

1.3 BACULOVIRUSES

Baculoviruses are double-stranded DNA viruses that have only ever been isolated in insects and other arthropods. Although they occur widely in the Lepidoptera (moths and butterflies), Hymenoptera (bees and wasps) and Diptera (flies), only a few have been characterised in detail (Rohrmann, 2013). However, baculoviruses in the Lepidoptera have been very well described at a molecular level and are very commonly used as model host-pathogen systems in the study of disease dynamics from a genetic to a physiological and population level (Cory and Myers, 2003). As baculoviruses play a role in regulating host population dynamics (Cory and Myers, 2003) (section 1.3.2), they have also been used as biological pesticides (Mishra, 1998).

Baculoviruses can be easily identified by their morphology under a light microscope and, in the Lepidoptera, by their characteristic pathology (Figure 1.1), and this in conjunction with molecular approaches has been used to classify two groups: nucleopolyhedroviruses (NPVs) and granuloviruses (GVs). Nucleopolyhedroviruses are relatively large (circa 1 - 5µm) and contain multiple virions within a protein matrix called an occlusion body (OBs), where occlusion bodies can be considered individual infectious units. Virions within OBs can contain either a single nucleocapsid (SNPVs) or multiple nucleocapsids (MNPVs). Granuloviruses are smaller (600 – 800 nm) and only contain one virion with one nucleocapsid. Phylogenetically, the baculoviruses form four different groups: the *Alphabaculoviruses* (NPVs) and *Betabaculoviruses* (GVs), which are specific to the Lepidoptera; *Gammabaculoviruses*, which are NPVs found in the hymenoptera; and *Deltabaculoviruses*, which are specific to the Diptera (Jehle et al., 2006, Rohrmann, 2013). Virus species are named after the host from which they were first isolated, for example isolates from the fall armyworm are *Spodoptera frugiperda* multiple nucleopolyhedrovirus or *Sf*MNPV. This can lead to some complexity, as virus species isolated from multiple different hosts can be closely related, sharing more than 95% of the same amino acid sequence, but having different names (Harrison and Bonning, 1999). In addition, isolates from the same host may be genetically very different but share the same name (Li et al., 2002).

Overall, baculoviruses are considered to be highly host-specific but this varies widely from NPVs that are known to infect only a single species (Barber et al., 1993) to *Autographa californica* MNPV (*Ac*MNPV), which is found in up to 15 different lepidopteran families (Cory and Myers, 2003), although these data come from laboratory studies and host range may be lower in the field owing to overlaps in spatial and temporal synchrony. Given the relative specificity of the baculoviruses, we might expect close co-evolutionary relationships with their hosts. However, host range does not generally follow taxonomy and pathogenicity also varies widely across host species, making this difficult to predict (Cory and Myers, 2003).

Figure 1.1: Characteristics of baculovirus pathology. Insects infected with baculoviruses have very characteristic mortality traits. Insects demonstrate positive phototaxis (movement towards the light) and as such are often found in the upper parts of the host plant, and can be easily identified by the characteristic v-shape of the cadaver (top). The exoskeleton of cadavers ruptures releasing occlusion bodies into the environment (middle). The distinctive spherical occlusion bodies are easily identified under a light microscope (bottom). Images attributable to Ken Wilson (top), James Solomon / Wikipedia (middle; https://en.wikipedia.org/wiki/File:CasualtyNPV.jpg) and the author (bottom)

1.3.1 DISEASE TRANSMISSION AND MIGRATORY DISPERSAL

Transmission is a fundamental aspect of disease ecology, and this is particularly relevant within the context of migratory disease ecology as the pathogen must be able to adapt to spatial and temporal fluxes in host population density that occur throughout the migratory season. In the baculoviruses, disease transmission occurs in two ways: vertically (adult to offspring) and horizontally (larvae to larvae).

Horizontal transmission

Horizontal (larvae to larvae) transmission is through to be the primary means of infection in most baculovirus systems (Cory and Myers, 2003). Larvae become infected when they ingest viral occlusion bodies (OBs). These OBs (Figure 1.1) are a very stable form of the virus. They are made up of individual virions contained in a protein matrix. This protein coating protects the virus from the external environment and enables the virions to remain infectious for many years (Thompson et al., 1981). When larvae encounter and ingest these OBs, the alkaline pH in the insect's gut breaks down the proteinaceous coat. This releases the individual virions which pass through the peritrophic membrane and enter the insect's system. Where the virus successfully overcomes the cell wall defences, DNA nucleocapsids are released into the cell and initiate infection in the nucleus. In flies and sawflies, infection is limited to the midgut, but in the Lepidoptera it spreads to other tissues as a non-occluded budded virus (Rohrmann, 2013, Cory and Myers, 2003). Where infection is fatal in larvae, the body tissue is converted into occlusion bodies at the end of the infection cycle. This results in a characteristic pathology where the body tissue disintegrates (Figure 1.1) and occlusion bodies are spread back into the environment. Susceptibility to oral infection is well known to decrease with age (Teakle et al., 1986), possibly due to age-related rates of sloughing infected midgut cell, and there is no evidence to date that hosts modulate their feeding behaviour in order to reduce ingestion of occlusion bodies.

Horizontal transmission and migration

There are no records of horizontal transmission between adults and death due to horizontal transmission is only known to occur in the larval stage. As such, the primary contribution of horizontal infection to migratory populations is likely to be sublethal infection which is transferred vertically from migratory adults to their offspring (see vertical transmission and migration below). Perhaps more relevant is the variety in baculovirus strains that the offspring of a migratory adults encounter when moving between habitats. The soil is known to act as a long term reservoir of baculovirus occlusion bodies (Thompson et al., 1981), which can survive in the environment for many years (Fuxa, 2004) and so moving between habitats may expose insects to a wider pathogen diversity. However, OB stability varies depending on the habitat type. Specifically, occlusion

bodies are highly susceptible to UV degradation (Jaques, 1967) and in the tropics OB survival has been shown to be reduced to a few days (Grzywacz et al., 2008). As such, occlusion bodies are assumed to only persist for a short time on the surfaces of plants, which is where highly susceptible early instar larvae are mostly likely to encounter them. Most insects also migrate to avoid unsuitable winter conditions. As such high levels of sunshine in the habitats associated with migration might reduce exposure for migrants versus residents or insects in tropical areas.

Relatively little is known about how pathogen dispersal works at the micro-habitat scale, but evidence suggests host dispersal (Goulson, 1997) and weather events might play a role (Fuxa and Richter, 2001), with rainfall linked to epizootics in some instances (Fuxa and Geaghan, 1983). This is particularly relevant in the tropics and for species such as the African armyworm *Spodoptera exempta*, where baculovirus epizootics are commonly associated with the large populations that migrate to follow the summer rains. Birds, farm machinery and other insect species are implicated in the spread of baculoviruses (Fuxa, 2004). Indeed, in the gypsy moth, *Lymantria dispar*, which is invasive in North America, the baculovirus that plays a key role in regulating host populations was thought to have been introduced by parasitoids (Myers and Cory, 2016). As such, migratory parasitoids that follow their hosts may contribute to the largescale movement of associated pathogens. Although it is only known to occur in larvae and as such may not influence long distance migration, it is interesting to note that baculoviruses do influence the dispersal behaviour of larval hosts. Particularly well studied is the tendency of infected larvae to become hyper-active and engage in positive phototaxis (movement towards the light, Figure 1.1). This is assumed to aid dispersal of the virus into the environment, and has to some extent been associated with the protein tyrosine phosphate (*ptp*) in some baculovirus species (van Houte et al., 2012).

Vertical transmission

Vertical transmission encompasses the "passage of virus from the adult to their progeny by any means" (Cory and Myers, 2003), although in the baculoviruses it is commonly thought to be the result of either transovum (surface contamination of the eggs) or transovarial transmission (virus contained within the egg). Infection by this means can be very high, with up to 50% of the progeny infected (Kukan, 1999) and can be passed on by both sexes (Burden et al., 2002). If the virus is truly latent (non-infecting and non-replicating) has in the past proven difficult to establish, but work to date suggests that the virus is not truly latent but rather replicating at low levels (Vilaplana et al., 2010, Hughes et al., 1997, Burden et al., 2002). Vertical transmission may also result as an effect of horizontal transmission in the parental generation, although the outcome of such infection seems to be highly variable. For example, in a laboratory study of the beet armyworm, *S. exigua*, virus challenge resulted vertically-transmitted infection in anything from

15% to 100% of the population depending on the magnitude of the challenge the insects received and the larval instar that was infected (Cabodevilla et al., 2011a, Murillo et al., 2011). This sublethal infection persisted for five generations and the covert disease dynamics varied over the course of the host's life cycle. The level of infection and virus transcription also varies with the host's life history and seems to be highest in the larval stage (Cabodevilla et al., 2011a, Murillo et al., 2011, Graham et al., 2015).

Vertical transmission and migration

As only adult moths are capable of long distance flight, and reaching the adult stage requires larvae to survive infection, it seems natural to assume that the vertical transmission would play an essential role in migratory host populations. However, the disease dynamics are unlikely to be this simple as if a pathogen relies on vertical transmission alone, it needs to be 100% efficient, infecting all host offspring. If this is not the case, prevalence will decrease with every generation and the pathogen will eventually become extinct (Cory, 2015). Detecting the extent to which vertical transmission contributes to such migratory disease dynamics is difficult to determine, especially in field populations. However, work to date would suggest that while the probability of detecting covert infection in populations is relatively high, virus prevalence is very variable. In the African armyworm, *S. exempta*, almost all field-caught insects carried the virus (Graham et al., 2015), but the virus is not always transcriptionally active (Redman et al., 2010). In western tent caterpillar, *Malacosoma californicum*, pathogen levels were fluctuated between generations (Myers and Cory, 2016), while in the cabbage moth, *Mamestra brassicae*, covert virus infections were present at all field sites, but the level of infection within field sites varied from 50% - 100% (Burden et al., 2003). As such, the evidence for 100% efficiency in vertical transmission is weak and the presence of horizontal transmission in the baculoviruses would seem suggest that both transmission modes play an essential part in a mixed strategy. As horizontal transmission requires death at the larval stage and vertical transmission relies on adult survival and reproduction, the two strategies may equally be considered to conflict (Cory, 2015) and there is some evidence that horizontally-transmitted isolates are more virulent (Cabodevilla et al., 2011a). However, vertical transmission is likely to play an important role in enabling the pathogen to persist in conditions that are not conducive to horizontal transmission, for example low population densities, life stages that are not susceptible to infection-induced mortality or, as is relevant to this thesis, during migration.

but virus load was also found to differ in different body regions, being highest in parts of the body with very low lipid content (head, wings and legs) and lowest in the parts of the body where most of the energy for flight is either stored or consumed (abdomen and thorax) (Graham et al., 2015) (Dingle, 2014).

1.3.2 EPIZOOTICS AND THE EFFECT OF BACULOVIRUSES ON POPULATION DYNAMICS

Bringing together these two modes of baculovirus transmission and trying to understand how they contribute to fluctuations in pathogen disease-dynamics is still an open question, which has only been well studied in a small number of species (Myers and Cory, 2016).

The occasional occurrence of baculovirus epizootics in noctuid genera including *Spodoptera*, *Heliothis* and *Helicoverpa* has made them of particular interest as sources of biological pesticides (Cory, 2003). However, long-term studies of epizootics and what drives them are rare, and where baculovirus disease dynamics have been studied in the field, the focus is on horizontal transmission and death at the larval stage. Field trials investigating sublethal effects in the adult population are also rare, and little is known about how vertical transmission might contribute to Lepidoptera-baculovirus systems. Yet this seems highly relevant, as spontaneous outbreaks of NPV in otherwise healthy populations have been observed for more than 100 years. How this relates to vertical transmission and possible covert infections is still not entirely clear, but various 'stressors' that may induce these outbreaks have been investigated, including population density, temperature and humidity, diet and infection with a second pathogen (Cory and Myers, 2003).

One system where this has been well studied is the western tent caterpillar, *Malacosoma californicum pluviale*. Native to North America, females lay eggs in family batches, which feed gregariously in large silken tents. Well known for its cyclical population dynamics, which are to some extent regulated by the baculovirus *Mcpl*MNPV, the species is not migratory (Myers and Cory, 2016). However, there is very little genetic structure in populations that cover several hundred kilometres (Franklin et al., 2014, Franklin et al., 2012), suggesting that females are capable of long distance flight. By contrast, the structure of the virus population is hierarchical: isolates in the order of family, then population and across islands. The population genetic structure also does not seem to vary significantly between population peaks and troughs, suggesting that there is no loss of population level genetic variation within these outbreaks, which in turn suggests there is no direct link between this neutral genetic variation and disease resistance. Levels of vertical transmission in this system seem to be relatively low, with only 4-9% of eggs infected in high-density populations. Covert viral loads however seem to peak prior to an outbreak, and interestingly there is a large amount of variation in susceptibility both within

families and across sites, such that insects which haven't recently experienced an epizootic are more susceptible to infection (Cory and Myers, 2009, Myers and Cory, 2016).

A second species in which the disease dynamics have been relatively well studied is the African armyworm. Available evidence suggests that *S. exempta* is an obligate migrant, which exists in low population densities along the east coast of Africa in the dry season. Migration events occur primarily in response to rainfall, with insects using converging winds to move further inland, resulting in large population influxes in the consecutive generations (Rose et al., 2000). Outbreaks of the associated baculovirus, *Spex*NPV, increase through the migratory season and this is associated with an increase in baculovirus genetic diversity (Graham and Wilson, unpublished data). This species also exhibits phase polyphenism (multiple phenotypes) which exist on a continuum depending on the level of intra-specific crowding (Rose et al., 2000). At low population densities, individuals exhibit *solitaria* characteristics. These larvae are typically cryptically coloured and sluggish, and a reduced dispersal distance has been recorded in adults during tethered flight. In outbreak populations larvae enter a *gregaria* phase, becoming active, voracious feeders that are conspicuously darker in colour, and have an increased propensity to engage in long flights in the laboratory (Parker and Gatehouse, 1985a, Parker and Gatehouse, 1985b, Woodrow et al., 1987). This phase polyphenism has also been found to confer higher levels of resistance to *S. exempta* NPV larvae developing under crowded conditions, in the form of increased melanisation and phenoloxidase activity (Wilson et al., 2001, Wilson and Reeson, 1998). Referred to as density-dependant prophylaxis (DPP), solitary-phase larvae have higher levels of mortality when challenged with a baculovirus and vertical transmission is lower for larvae reared gregariously (Reeson et al., 2000), although it appears that this comes at a cost (Vilaplana et al., 2008).

1.3.3 BACULOVIRUS DIVERSITY

Baculoviruses in general, and MNPVs isolated from noctuids in particular, are known to be highly variable at a variety of levels (Cory and Myers, 2003). Genetically, baculoviruses replicate clonally but widely exchange material with different organisms, from other virus genotypes to the host and other co-infecting organisms. For exchange between genotypes, both isolates need to infect the same insect cell (co-occlusion), but this is common (Bull et al., 2001) and, between closely related genotypes, recombination can be up to 40% (Croizier and Ribeiro, 1992, Hajos et al., 2000). MNPVs in particular have very high levels of variability, and OBs can contain genetically-distinguishable genotypes (Simon et al., 2004a). Indeed, a single insect rarely contains a single genetic variant and this high level of diversity may contribute to the wide host ranges observed in MNPVs (Cory and Myers, 2003).

This level of genetic variation results in a wide variety of phenotypes that can alter the level of pathogenicity, the speed of kill and the extent to which larval cadavers disintegrate (Cabodevilla et al., 2009, Barrera et al., 2013, Barrera et al., 2011). Of interest are so called 'parasitic genotypes', isolates only capable of orally infecting a host when other genotypes are also present in the occlusion body (Lopez-Ferber et al., 2003, Simon et al., 2004a). Such genotypes are highly abundant and can affect the overall disease dynamics, leading to either reduction (Muñoz and Caballero, 2000) or increases in pathogenicity and changes in phenotype (Lopez-Ferber et al., 2003).

At a geographic scale, isolates have been shown to have very different genetic profiles (Gettig and McCarthy, 1982, Shapiro et al., 1991, Takatsuka et al., 2003) but what maintains this level of variation and what it's role might be is largely unknown, with very little information about how regional and temporal differences in genetic and phenotypic diversity relate to host-pathogen dynamics (although see Cory and Myers, 2009, Franklin et al., 2014, Franklin et al., 2012).

1.4 HOST PATHOGEN SYSTEM

In conjunction with its high reproductive rate and relatively short generation time, the wide host range and migratory capacity of the fall armyworm make it a highly successful colonising species (Sparks, 1979, Johnson, 1987, Luginbill, 1928). These features also make it an important agricultural pest of graminaceous crops such as corn, particularly as populations can be resistant to a variety of pesticides including pyrethroids, organophosphates, carbamates (Yu, 1991) and, more recently, the *Bacillus thuringiensis* (Bt) toxins in transgenic crops (Huang et al., 2014). As such, incorporating biological control agents into the pest management strategies for this species is very relevant and *Sf*MNPV has been widely investigated as a means of control (Pedrini et al., 2004) (Farrar et al., 2004).

1.4.1 THE FALL ARMYWORM, *SPODOPTERA FRUGIPERDA* (J.E. SMITH)

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) is highly polyphagous, known to feed on more than 60 different species of host plant from 20 different families, with a preference for graminaceous hosts (Luginbill, 1928). It has a standard lepidopteran life cycle of egg, larvae, pupa and adult moth but the life history parameters of these different phases can vary widely, particularly in relation to temperature (Luginbill, 1928). On average, adults live for approximately two weeks and have highly variable levels of fecundity (Luginbill, 1928). The oogenesis flight syndrome hypothesis has not been directly tested in *S. frugiperda*, but is assumed to be present to some extent as females only oviposit approximately 3.5 days after emergence, and can continue laying for between 4 and 17 days (Sorge et al., 2000, Luginbill, 1928). The standard development time for eggs is two days, although this can take up to 11 days in some instances, and larvae go through five to six larval instars over a period of 10 – 13 days. Pupation on average takes an additional 9 days, with a maximum observation of 14 days. This amounts to an average development time of approximately 4 weeks (Luginbill, 1928), although in the winter a period of up to 90 days has been recorded in Texas (Vickery, 1929).

Rice and Corn Host Strains

Spodoptera frugiperda is a species where the use of genetic markers has been particularly successful in defining both host strains and migratory pathways (Nagoshi and Meagher, 2008). Two different haplotypes have been identified in the mitochondrial cytochrome oxidase I (COI) gene, which allows the fall armyworm to be broadly divided into two strain-specific groups. As these haplotypes are predominantly found in different host plants, they are referred to as corn-strain (CS; preferential association with large grasses such as corn and sorghum) and rice-strain (RS; pasture and turf grasses) (Pashley, 1989, Meagher and Nagoshi, 2013, Nagoshi et al., 2007c). This difference between strains is best documented in North American populations, although

studies suggest populations with the same strain-specific mitochondrial markers can be used to differentiate populations in Brazil with very high levels of accuracy, which suggests this is a relatively ancient divergence (Nagoshi et al., 2007c) (Lewter et al., 2006). Directional inter-strain mating biases would certainly suggest that opportunities for hybridisation are limited (Meagher and Nagoshi, 2013) (Pashley and Martin, 1987), although there is some evidence for putative hybrid sub-populations (Nagoshi and Meagher, 2008). Morphologically, the two strains are identical and no significant differences have been found in behaviour or physiology (Nagoshi and Meagher, 2008). However, the two strains exhibit different responses to their host plant, in particular variation in egg viability, larval development and even different levels of susceptibility to some pesticides (Nagoshi and Meagher, 2008).

Migration in North America

Present across South and Central America, the fall armyworm regularly migrates into Canada and has been recorded as far south as Argentina (Johnson, 1987) (Figure 1.2). There have also been recently been incursions into West Africa (Goergen et al., 2016), although it is not known if these are a result of migration or human-related activity. *Spodoptera frugiperda* cannot withstand the freezing temperatures that occur in the winter months in northern part of its range. As such it is not known to diapause but instead over winters at latitudes south of Florida and Texas (Luginbill, 1928) where generations are continuous all year round (Andrews, 1980). When ephemeral habitat become available, the fall armyworm migrates to more northerly latitudes (Johnson, 1987, Luginbill, 1928). For this reason, incursions into Canada and the northern United States can be recorded as migratory events with a high degree of certainty, and the species' migration is best understood in this part of the world. By contrast, very little is known about the migratory dynamics in the southern part of the species' range, and in the tropics migration may potentially be driven by wet and dry seasons in rather than temporal gradients (Johnson, 1987). For this reason, this thesis focuses on the migration of the fall armyworm in North America which is where all samples were collected.

In North America, insect migration is well known to be related to weather and climate (Johnson, 1995), which in turn is linked to the underlying geography. From Texas to northern Canada, the mid-latitudes of Northern America are highly seasonal with warm summers and below freezing winters. Synoptic (large) scale weather patterns are particularly influenced by the Appalachian mountain range in the east and the Sierra Nevada and Rocky mountain ranges on the west coast. Between the two lie the Great Plains and the Mississippi River Drainage Basin. Both are large, flat, rolling plains that run from Texas in the south to Alberta, Canada in the north, with the result that there is little restriction to air movement, which moves relatively freely from the Gulf of Mexico

in the south to the arctic in the north and winds in this region are commonly used by many North American migrant species.

In the spring and mid-summer, the weather is mild and temperatures across continental America range between 20°C and 30°C (Johnson, 1995), well within the 15°C - 35°C optimal temperature threshold for this species (Luginbill, 1928). The pole-ward advance of warm weather gives insects such as *S. frugiperda* the opportunity to move north and take advantage of seasonal habitat which can support reproduction and survival. In the spring, warm southerly air drawn northwards into the Mississippi River Drainage Basin and high speed winds that form low level jets are used for insect migration (Johnson, 1995), although depressions (areas of low pressure often accompanied by wet, windy weather) are likely to have some effect on the trajectory and deposition sites available to these insect migrants. Into late spring and summer, these weather patterns persist, but the winds are weaker and the distances covered by migrants are likely to be smaller. In the summer, the Bermuda high (a semi-permanent area of high pressure) offers additional opportunities for migration.

In the autumn, this pattern reverses. Habitat availability decreases and with severe winter temperatures that drop below freezing, insects must either return or perish. Equatorward winds that would enable a return migration do exist. In particular, there is likely to be increased opportunities for migration following a cold front, where winds in excess of 30 km/h can lead to downwind displacement distances of up to 300 km a night (Muller, 1985). As the weather cools, however, so the air temperature drops and later into the season temperatures may only support insect flight for a period of a few hours. Past October, there are probably very few opportunities for insect migration (Johnson, 1995). This is particularly relevant to trap catch data described in Chapter 5

This geography and its effect on the winds used for migration appears to have had a direct impact on the underlying population dynamics of the fall armyworm. Within the corn-strain, recent work by Nagoshi et al. (2012) has found single-base polymorphisms at two different sites on the COI gene. These COI haplotypes can be divided into four different sub-groups (CS-h1 to CS-h4, Nagoshi et al., 2007b) and the proportion of two of these sub-groups (CS-h4 and CS-h2; known as the h4/h2 ratio) have then been found to differ regionally. Specifically, they have been used to distinguish two genetically distinct overwintering populations, one in Texas with an h4/h2 ratio that is consistently less than 0.5, and another in Florida with an h4/h2 ration that is above 1.5 (Nagoshi et al., 2008). Recent work has used these different haplotype ratios to differentiate two separate migratory pathways, showing that the haplotype ratios are maintained either side of the Appalachian Mountains, with some mixing to the north and south of this mountain range (Figure 1.3) (Nagoshi et al., 2012). This has considerably advanced the field, which for a long time

did not improve upon the low-resolution description in Luginbill (1928), and assumed consistent genetic mixing across *S. frugiperda* populations in North America (Johnson, 1987).

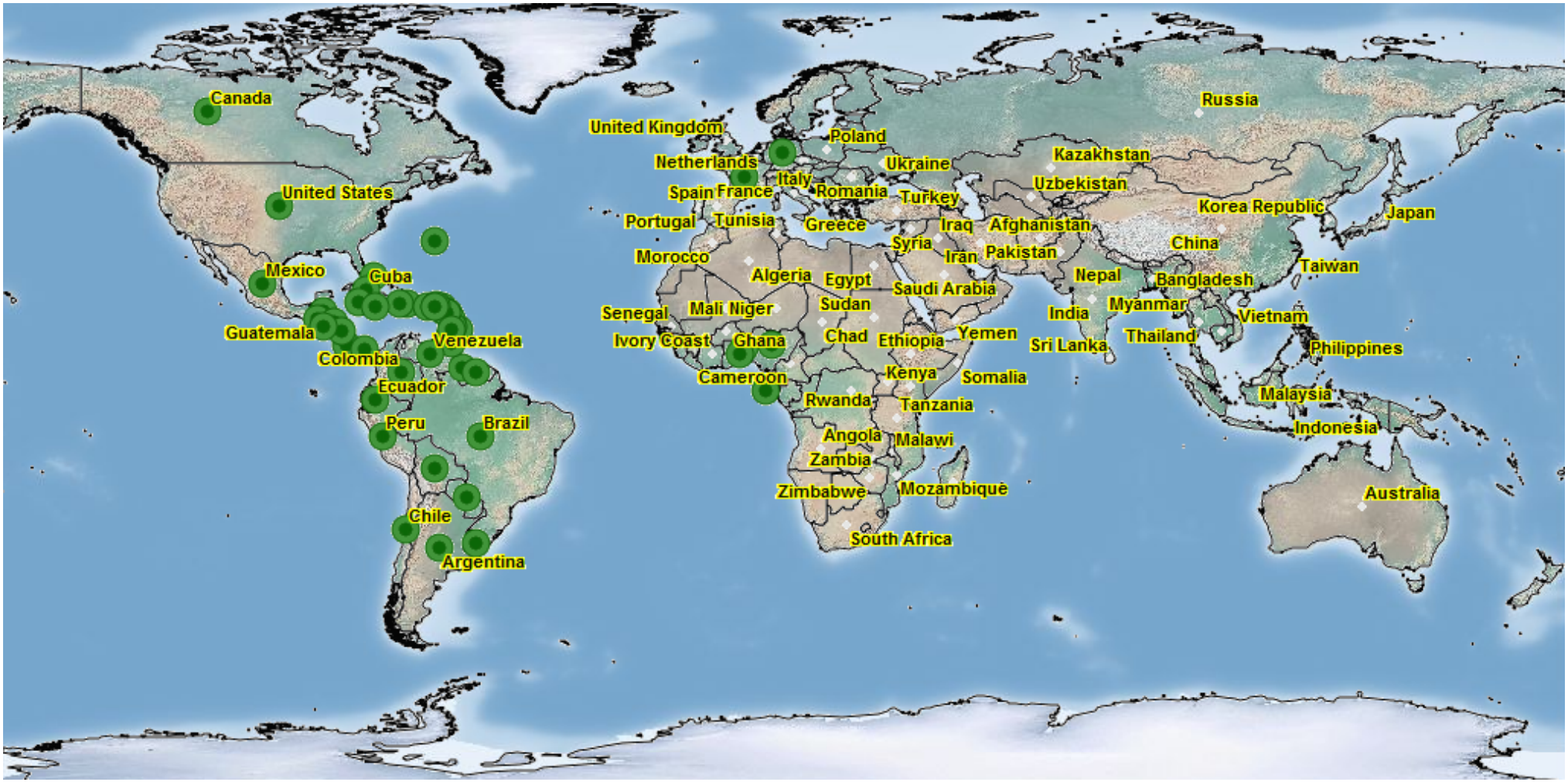

Figure 1.2: Fall armyworm distribution. The distribution of fall armyworm across the Americas, Africa and Europe (EPPO, 2016). The species is native to northern and southern America, and invasive in West Africa where it has only been recorded within the last two years. Presence in Europe is documented but likely to be due to trade imports and the species is not known to survive in this region.

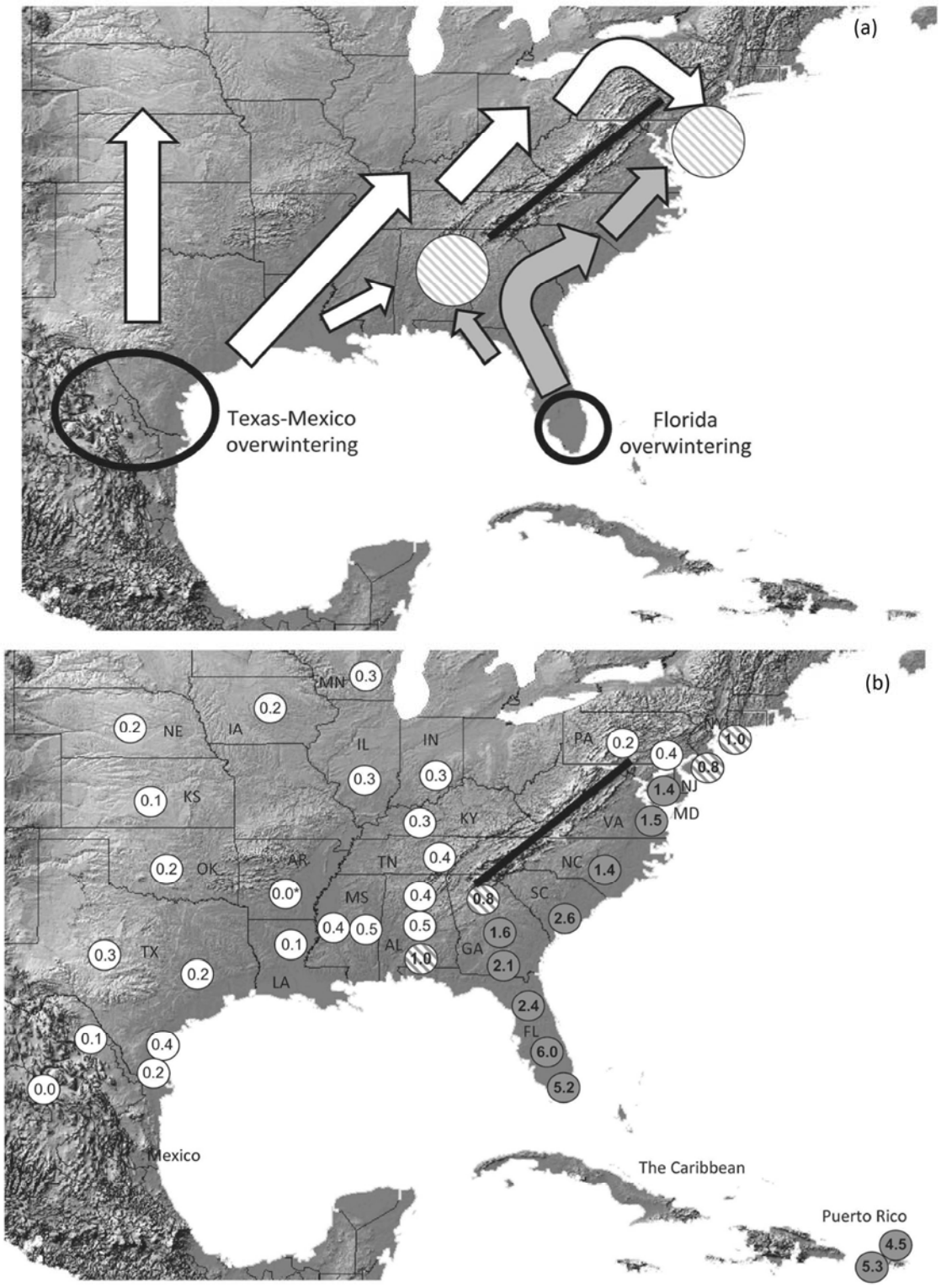

Figure 1.3: Fall armyworm migratory routes and haplotype distributions. Recent work has demonstrated how variation in haplotype ratios wither side of the Appalachian Mountain Range in the United States of America (b) and how this translates into two separate migratory populations based on overwintering source populations (a). Circles with lines show areas of overlap in haplotype ratios, and the approximate location of the Appalachian Mountain Range is marked with a diagonal black line. Image source: Nagoshi et al., 2012

1.4.2 SPODOPTERA FRUGIPERDA MULTIPLE NUCLEOPOLYHEDROVIRUS (*SF*MNPV)

The fall armyworm is infected by two baculovirus species; *Sf*MNPV and *S. frugiperda* granulovirus (*Sf*GV). Less is known about *sf*GV, although the genome was recently sequenced (Cuartas et al., 2015). The pathogen used in this thesis is a wild-type strain of *Sf*MNPV isolated in Nicaragua (*Sf*NIC), which is described in detail below.

Host specificity

Within the *Alphabaculoviruses* there is evidence for three different subclades, designated A, B and C. *Spodoptera frugiperda* MNPV falls within subgroup A, and clusters closely with NPVs from the closely-related species *S. exigua* (the Beet armyworm) and *S. litura* (Oriental leafworm moth) as well as the Bertha armyworm *Mamestra configurata* NPV (Bulach et al., 1999). All three occur in the Americas, although the Oriental leafworm moth is an old world species and a recent introduction in the USA (EPPO, 2014). Phylogenetic analysis and genetic studies would suggest *Sf*MNPV is closely related to *Mamestra configurata* NPV (Tumilasci et al., 2003) and *S. exigua* MNPV (*Se*MNPV) (Harrison et al., 2008, Wolff et al., 2008). In particular, *Se*MNPV and *Sf*MNPV share gene arrangements and sequence identities of over 65% and work to date suggests that they may have evolved from a common ancestor (Simon et al., 2005). Recombination between these different virus species is also known to occur. For example, there is evidence of recent horizontal gene transfer between two open-reading frames, ORF023 and ORF024, in a Columbian strain of SfMNPV, acquired via recombination with an NPV from the Oriental leafworm moth, *S. litura* (Patricia Barrera et al., 2015).

Where they have been tested, none of the NPVs isolated from outside the genus *Spodoptera* were able to induce an LC_{50} (the lethal concentration at which there is 50% mortality) (Shapiro and Hamm, 1999). Nucleopolyhedroviruses isolated from *S. littoralis* and *S. exigua* however do produce fatal disease in the fall armyworm, although the level of gene expression for *S. exigua* MNPV was much lower and infection was very nearly cleared. This was true for both oral and injected infectivity assays, suggesting resistance is not only in the midgut. Interestingly, *Sf*MNPV is also fatal to the heterologous hosts of these viruses, but it fails to produce OBs (Simon et al., 2004b).

Geographic variation

There are very few studies that have attempted to describe how baculovirus populations respond to the migratory nature of their hosts, and only one which looks at changes in *Sf*MNPV pathogenicity in relation to migration of the fall armyworm (Fuxa, 1987). In this study, the proportion of insects that were susceptible to overt infection was highly variable. Different virus isolates caused different levels of overt infection in depending on the geographic source

population of the host and in some instances, these relationships suggested that the greater the geographic distance between host population and pathogen isolate, the higher the levels of mortality. However, these relationships were very dynamic and fluctuated significantly over time. Source location wasn't always a significant predictor of mortality and the level of infection caused by a particular isolate wouldn't always result in the same level of mortality when tested in the same host population a year later (Fuxa, 1987). Given recent insights into fall armyworm migratory patterns (Nagoshi et al., 2012) it is worth noting that the response to infection with a particular isolate clustered separately for the two overwintering locations of Florida and Texas. These responses again differed to those in host populations from Mexico and Brazil, suggesting that although there are high levels of inter-annual variability there may be some geographic trends in susceptibility but that these are very variable and fluctuate over time.

While few studies attempt to relate *Sf*MNPV infection to migration in the host, more work has been done on quantifying genetic variation in virus isolates at a variety of different spatial scales. Most of these studies relate to areas in South America, where less is known about the migratory behaviour of *S. frugiperda*, but research still provides an interesting insight into possible patterns of variation. For example Rowley et al. (2010) found a distinct North American clade that separated from isolates collected in Columbia, although interestingly the isolate collected in Florida was very similar to that from Columbia. Yet even within a single geographic population, isolates taken from different larvae within the same field show a very high level of sequence divergence, suggesting very different dynamics to the hierarchical structure observed in the Western Tent Caterpillar (Cory and Myers, 2009, Franklin et al., 2014). Despite this level of genetic variation, bioassays suggest that this level of host diversity results in very little biological variation in pathogenicity (Rowley et al., 2010). Where changes in phenotype do occur, it seems that they may be driven by very small changes to localised regions of the genome in isolates that are otherwise show very little genetic differentiation (Barrera et al., 2011) (Crook, 1981). Other studies that have compared *Sf*NIC (specifically the *Sf*MNPV-B genotype) with isolates from the United States (*Sf*MNPV-3AP2) and Brazil (*Sf*MNPV-19) again found that the nucleotide sequences were highly conserved (99.35% identity), with positive ("directional") selection pressures acting on three open-reading frames (ORFs: Sf49, *pif*-3; *sf57, odv-e66b* & sf122, unknown function). Negative ("purifying") selection was implied for most other ORFs (Simon et al., 2011).

In conclusion, while there are regional differences in *Sf*MNPV isolates, what drives these differences and how they relate to the host is still somewhat of an open-ended question, but it is possible that the variation in phenotype is driven by very small changes to parts of the genome essential for infectivity, such as transmission (Cabodevilla et al., 2011a, Simon et al., 2004a).

1.5 THE INTERACTION BETWEEN MIGRATION AND DISEASE

Migratory disease ecology focuses specifically on the study of pathogen evolution and disease dynamics in response to regular, seasonal mass movements of populations (Altizer et al., 2011). Primarily, these disease dynamics are driven by pathogen virulence and host susceptibility (Myers and Cory, 2016) and this section begins by defining each of these terms within the context of migration (section 1.5.1). Section 1.5.2 then describes how pathogen virulence and host susceptibility can be altered by a combination of biotic and abiotic factors, while sections 1.5.3 to 1.5.6 focuses on five mechanisms that have been predominantly discussed in the literature which specifically relate to how the act of migration may influence geographic disease dynamics. These five mechanisms are *migratory spread*, *migratory culling and escape*, *migratory recovery* and *migratory exposure*. Section 1.5.7 describes what is known of the relationship between migration and disease in insects, while section 1.5.8 describes the primary theoretical background for the specific hypotheses tested in this thesis (section 1.6).

1.5.1 DEFINITIONS

The outcome of infection in a single individual is determined by the interplay between the virulence of the pathogen and the susceptibility of the host. In this thesis, virulence and susceptibility are defined as follows:

Pathogen Virulence is the severity of a disease (Schmid Hempel, 2011). The most common way of quantifying pathogen virulence is the level of mortality in a population. However, as this thesis focuses on sub-lethal effects, pathogen virulence is primarily considered in terms of reductions in host fitness. In this thesis, I have specifically focused on host development time, reductions in flight capacity and weight, and changes in wing length as these are all linked with the migratory syndrome (Dingle, 2014).

Host Susceptibility is the failure to resist infection (Schmid Hempel, 2011). This commonly considered either in terms of either host tolerance or host resistance. In this thesis, tolerance and resistance are defined in line with (Råberg et al., 2007):

> Tolerance: The capacity of the host to tolerate infection, with impact on fitness or associated fitness costs. This is the inverse response of pathogen virulence, and uses similar means of quantification.
> Resistance: The ability of the host to reduce the level of infection. In this thesis, resistance is quantified in terms of viral copy number, which reflects the number of virions within a host (Graham et al., 2015).

The variation of host tolerance and pathogen virulence within a population will determine the disease dynamics of an infection. How these disease dynamics vary at spatial scales is dependent on the movement of both host and pathogen in space, and the extent to which the two populations maintain spatial synchrony (Bauer et al., 2016).

This thesis focuses on how host susceptibility and pathogen virulence are affected by the behaviours and physiological adaptions that are part of the migratory syndrome. For this reason, I have used two new terms to describe the interactions between host susceptibility, pathogen virulence and migratory symptoms:

Migratory tolerance: As tolerance and virulence are both measured in terms of in host fitness, I have used the term *migratory tolerance* to describe how disease affects host fitness in terms of the behavioural, physiological or genetic adaptions linked with the migratory syndrome (Dingle, 2014)

Migratory resistance: I have used the phrase *migratory resistance* to describe how the behavioural, physiological or genetic adaptions linked with the migratory syndrome (Dingle, 2014) affect changes in the level of pathogen infection (specifically virus load, (Graham et al., 2015))

These two terms specifically relate to the graphs in Figure 1.4, and the hypotheses tested in Chapters 3 & 4 (see section 1.6).(Lively et al., 2014)

1.5.2 BIOTIC AND ABIOTIC INFLUENCES ON MIGRATORY DISEASE DYNAMICS

The disease literature to date has shown that the outcome of infection in migratory populations varies between taxa and depends upon a wide range of biotic and abiotic factors such as weather and climate (Hill et al., 2016, Pérez-Rodríguez et al., 2013), life history traits (Hannon et al., 2016) and circadian rhythms (Martinez-Bakker and Helm, 2015). Biotic factors which are especially relevant to migratory-disease interactions are the behavioural, physiological and genetic adaptations that form the migratory syndrome (and specifically how the diversity of this phenotype within the host population) affects the susceptibility, virulence and subsequent spread of infectious disease (Lively et al., 2014). In migratory systems, another key consideration is how host and pathogen maintain spatial and temporal synchrony, particularly in terms of ephemeral habitats, the co-occurrence of migratory populations and the timing/ geography of previous migratory events (Bauer et al., 2016). In this respect, the pathogen's transmission strategy is essential: how species-specific the pathogen is and whether or not it can exist outside the host (Lively et al., 2014) are traits that affect its ability to retain contact with the host population. Yet within the disease literature some of these aspects, such as the trade-off between generalism

and specificity, are still poorly understood (Woolhouse et al., 2001) and this limits our ability to draw broad inferences on migratory disease relationships across taxa.

Equally important is how the pathogen maintains the genetic diversity required to match polymorphic traits in the host population (Lively et al., 2014). This is something we might intuitively assume is driven by co-evolution or reciprocal selection with the host (Ebert and Hamilton, 1996), but detailed studies within the Lepidoptera would suggest that these host-pathogen relationships are highly context-dependent (Myers and Cory, 2016) and that even within the baculoviruses this is not a straightforward process, influenced by complex factors such as genotype interactions (Lopez-Ferber et al., 2003) and interspecific competition also affecting disease outcome (Escribano et al., 2001).

At the level of the host, the presence of pathogens raises the question of disease as a driver of migration (section 1.2.1) (Altizer et al., 2011). The extent to which this occurs is still largely unknown but it is likely to require a balance between investment in immunity (Schmid-Hempel, 2005) and migration (Chapman et al., 2015b), both of which are known to be very costly. These interactions are likely to result in selective pressures for both the host and the pathogen, and to date the literature provides evidence for a variety of different relationships between migration and disease, which are discussed below.

1.5.3 Migratory spread

For many years, the standard assumption of disease in migratory host-pathogen systems was migratory spread, where movement of the host would result in the geographic dispersal of the pathogen by the host (Altizer et al., 2011). This does occur. For example, there is some evidence that migration patterns of female little brown bats (*Myotis lucifugus*) influence the spread of the white-nose syndrome, an emerging fungal disease caused by *Pseudogymnoascus destructans*, which is capable of devastating local populations (Miller-Butterworth et al., 2014). Migratory birds have also been shown to be carriers of diseases such as West Nile virus (Rappole et al., 2000), while the migratory movement of saiga antelope has been linked to geographic prevalence of abommasal nematodes (Morgan et al., 2007).

While the presence of disease in a migrating population is clear evidence for the spread of that pathogen is a result of host movement, it must be kept in mind that migrants may have complex co-evolutionary relationships with their pathogens. As such, the disease dynamics that result from spatial spread will depend on a wide variety of factors, particularly host susceptibility. For emerging pathogens such as white-nose syndrome in bats (Blehert et al., 2009) or chytridiomycosis in amphibians (Roznik and Alford, 2015), the spread of disease as a result of long-distance movement may have devastating consequences. In the literature, however, such

virulent isolates seem to be exceptions and, given how energetically demanding migration can be (Bonte et al., 2012), it might be reasonable to assume reductions in virulence with increasing energetic investment (Osnas et al., 2015). It is perhaps more relevant to question how migration affects pathogen diversity in space and time, and how this contributes to epidemics. In outbreaks of the African armyworm, for example, increased diversity has been associated with higher levels of mortality (Chapman et al., 2015b, Myers and Cory, 2016, Graham et al., 2012), while migration along a North America migratory route leads to rapid genetic assortment and transportation of novel lineages of avian influenza virus (Fries et al., 2015). Particularly key in relation to human diseases is also the question of whether or not migrants may influence the spread of resistant genotypes (Bonnedahl et al., 2015).

1.5.4 MIGRATORY CULLING AND MIGRATORY ESCAPE

Two non-exclusive behavioural mechanisms which might lead to a decrease in pathogen prevalence with increased migratory distance are migratory culling and migratory escape (Altizer et al., 2011). Migratory culling describes a process whereby infected individuals are unable to undertake or complete migration, while migratory escape describes healthy individuals which escape diseased areas. Interestingly, where this has been tested theoretically, a third mechanism by which migration increases mortality in both susceptible and infected individuals was also found to reduce disease prevalence by simply reducing the number of susceptible individuals (and hence transmission rates) in a population (Shaw and Binning, 2016). This is best studied in the monarch butterfly (Altizer et al., 2011), but in recent years other studies in a wide variety of taxa have also associated reduced disease prevalence with increased dispersal distances (Moore and Brown, 2014, Qviller et al., 2013, Poulin et al., 2012), with some particularly interesting examples of migratory escape such as migratory allopatry (spatial and temporal separation between adult and immature hosts) (Krkošek et al., 2007). This relationship, as it relates to parasites, is particularly interesting as some parasites are known vectors of arboviruses (viruses transmitted by arthropods). This adds an additional trophic level to the spread and maintenance of disease in migratory populations, yet one where there is evidence of migratory escape driven by parasite behaviour. For example, one study on the prevalence of buggy creek virus in the ectoparasitic swallow bug *Oeciacus vicarious* found that dispersing ectoparasites had much lower levels of infection than those which remained in high density colonies of cliff swallows (Moore and Brown, 2014).

While this body of evidence shows that reduced parasite and disease loads certainly occur across a range of taxa, differentiating between this as an outcome of migration as opposed to a causal mechanism (i.e. a driver for migration) is much more difficult to establish, particularly as drivers may co-vary. For example, in the African armyworm, increased investment in immune function

has been shown at high population densities (Wilson et al., 2001) and this trait is closely associated with increased in flight capacity (Woodrow et al., 1987).

1.5.5 MIGRATORY RECOVERY

A third mechanism that would lead to decreases in disease prevalence with increasing migratory distance proposed by (Shaw and Binning, 2016) is migratory recovery. The authors describe this as a situation where individuals lose their parasites and recover from infection during migration as a result in changes in the host's biology (e.g. increases in immunity) or external environment (improved access to nutrients or forage during migration, for example). This was demonstrated theoretically only recently, but would perhaps be supported by other examples where migration has been shown to lead to an increase in overall fitness (Chapman et al., 2012). As this is such a recent development, there is currently little experimental work to test this hypothesis. However, some studies would suggest that movement to different habitats and micro-habitats might result in sub-optimal conditions for the pathogen, reducing prevalence overall. For example, the common mist frog, *Litoria rheocola*. Although a highly sedentary species, it was found that the increased distances the frog moved in the summer and their choice of microhabitat between seasons reduced chytridiomycosis infection because the host behaviour resulted in unfavourable conditions for the pathogen. This was reversed in the winter (Roznik and Alford, 2015). Across multiple bat species, there is also evidence that white nose syndrome is highly infectious in the winter, but reduced to almost zero prevalence in the summer, partially as a result of seasonal changes in the host's physiology, specifically hibernation (Langwig et al., 2015).

1.5.6 MIGRATORY EXPOSURE

Finally, another concept for which there is some evidence in the literature is migratory exposure (Hannon et al., 2016, Altizer et al., 2011): the idea that as animals migrate they are exposed to pathogens. This is particularly relevant for species where there is only partial migration and/or where the pathogen has a very wide host range. There is certainly evidence for this, particularly in birds. For example, in a meta-analysis of haematozoan parasite diversity in ducks, geese and swans, pathogen diversity was positively associated with migratory distance (Figuerola and Green, 2000). Additionally, phylogenetic analyses suggest that, in haemosporidian parasites with particularly broad species ranges, transmission between resident and migratory song bird hosts has occurred multiple times across the migratory ranges in Europe and Africa (Waldenstrom et al., 2002). Alternatively, where an overwintering ground or stopover site is used regularly, pathogens may adapt to this regular food source, as is observed in the Bogong moth *Agrotis infusa* which is parasitised by a mermithid nematode that occurs in the overwintering caves of the Australian Alps (Common, 1954).(Bartel et al., 2011, Brown and Shine, 2014, Pizzatto and

Shine, 2012, Dorhout et al., 2011, Lindsey and Altizer, 2009, Roznik and Alford, 2015, Altizer et al., 2006), Chapman et al. (2015b), (Bradley and Altizer, 2005, Dingle, 2014)

1.5.7 THE INTERACTION BETWEEN MIGRATION AND DISEASE IN INSECTS

While recent advances in this field have begun to describe the different geographic and temporal relationships observed in migratory host-pathogen systems, most of the migratory disease ecology literature relates to birds and mammals, with some examples in fish and amphibians. Except for extensive studies in North American populations of the monarch butterfly *Danaus plexippus* and its protozoan parasite, *Ophryocystis elektroscirrha*, there are very few studies of migratory host-pathogen systems in insects.

Host-parasite interactions in the monarch butterfly

Monarchs form well known resident and migratory populations in North America. Populations in eastern North America are highly migratory, undertaking the longest distance migration to Mexico in the fall. Western North American populations make the same migration but over a much shorter distance, while populations in Florida are sedentary. This variation makes the monarch an ideal study system for understanding parasite prevalence in relation to movement patterns and it is well established that in North America parasite prevalence is negatively associated with host dispersal distance (Altizer et al., 2000) (Bartel et al., 2011) (Satterfield et al., 2015) (Altizer et al., 2015). The differences in prevalence of *O. elektroscirrha* in these different populations is striking, with infection rates as low as 8% in highly migratory eastern North American populations, but increasing to 70% in sedentary populations in Florida, with an intermediary level of infection in eastern North American migrants (Altizer et al., 2000). *Ophryocystis elektroscirrha* is transmitted both vertically when females scatter parasites onto eggs and horizontally between mating adults. In line with expectations of maternal transmission three is little or no effect on host fitness when the parasite is horizontally transmitted by females or when horizontal transmission results in low to intermediary levels of infection (Altizer and Oberhauser, 1999). At highest levels of infection (circa 1000 spores per larva) (Altizer and Oberhauser, 1999) insects have been found to have smaller wing spans and lower body mass (Altizer and Oberhauser, 1999, Altizer, 2001) and this has been shown to affect flight performance (Bradley and Altizer, 2005). This in combination with studies of virulence that demonstrate that parasite fitness is maximised at intermediate levels of infection suggest a step function may best describe the migratory tolerance strategy of this host pathogen system (de Roode et al., 2008b, Chapman et al., 2015b) (Figure 1.4, section 1.5.8).

This reduction in parasite prevalence increases from early to late in the season (Bartel et al., 2011) as well as reductions in flight capacity (Bradley and Altizer, 2005) suggest the cost of high levels

of infection result in both migratory culling and migratory escape in this species. However, there are some interesting variations. For example, in year-round breeding populations in tropical climates such as Hawaii, where monarchs form a single breeding population, dispersal distance isn't significantly related to parasitism by *O. elektroscirrha* and there are very high levels of spatial heterogeneity in pathogen prevalence (Pierce et al., 2014). In addition, mating activity n overwintering locations is associated with higher parasite loads (Altizer and Oberhauser, 1999) but this doesn't seem to change the frequency of heavily parasitised migratory insects in overwintering populations such as Mexico (Altizer et al., 2000), leaving the question of how parasite prevalence affects return migrants fairly open, as migratory culling may lower the parasite virulence and host susceptibility of the parasite that migratory populations encounter in overwintering source areas (Altizer, 2001).

Other migratory host-pathogen insect systems

The other migratory host-pathogen system that has been studied in a reasonable amount of detail is the African armyworm *S. exempta* and its associated baculovirus *Spex*NPV In this species, but even in this species studies have focused on transmission strategies, population dynamics, and the role of melanisation and polyphenism in disease resistance (see sections 1.3.1 and 1.3.2), rather than the specific relationship between migration and disease. Where this has been studied, work to date shown an increase in virus load over the migratory season, which is commonly associated with large scale epizootics in the host population (section 1.3.2), but this dynamic is not stable and fluctuates annually (Graham, R. unpublished data). There remaining papers are single studies, and often focus on dispersive species rather than truly migratory ones. All work to date reliably shows a cost in dispersal capacity associated with infection (Dorhout et al., 2011, Overton et al., 2006, Simmons and Rogers, 1991) but that cost shows variation depending on the physiological factors of a populations. For example, in the European corn borer *Ostrinia nubilalis* when infected with the common microsporidian parasite *Nosema pyrausta*, as well as a single field study of the fall armyworm Work in the European corn-borer is limited to a single flight study (Dorhout et al., 2011), where there was a linear cost of *N. pyrausta* infection in males but not females. However, when the data was pooled across moderate and heavily infected individuals, analyses suggested that step function observed in the monarch butterfly may also be evident in both sexes of the European Corn Borer. Interestingly however, this only affected flight in pre-reproductive period; from three days' post infection, the distances flown between control and infected groups were similar. Whether the differences in distance flown between newly emerged and 3 day old moths were significantly different wasn't stated, but the assumption here may be that moths reduce their flight distance with age (in line with the oogenesis flight syndrome, see section 1.2) and it is only long distance migratory flight that is limited by infection.

The only study in the fall armyworm to date is by Simmons and Rogers (1991) who looked at the prevalence of the ecto-parasitic nematode *Noctuidonema guyanenese* across the eastern United States. This study supported the mechanism of migratory spread (assumed to be standard for migratory disease dynamics in the period when the paper was published, see section 1.5.3), with low levels of pathogen prevalence in early migratory populations that increased through the summer months.

Bateman's principle in migratory insect species

Sex-biased dispersal, where the dispersive tendency of one sex is greater than the other, is a well-known phenomenon in birds and mammals (Miller et al., 2011). But how this relates to Bateman's principle of differing immune costs in different sexes (Nunn et al., 2009, Rolff, 2002) and the extent to which it is evident in insects hasn't been studied extensively in insects. Flight mill studies however, would suggest that differences in flight tendencies between the sexes do exist in migratory insect species (Dorhout et al., 2011, Bradley and Altizer, 2005, Davis et al., 2012, Gatehouse and Hackett, 1980) and there is evidence that infection can lead to significant variation in immune response in both males and females, which can affect flight characteristics. For example there is (Altizer et al., 2000{Bartel, 2011 #280, Satterfield et al., 2015, Altizer et al., 2015, Pierce et al., 2014)(Bradley and Altizer, 2005, Altizer et al., 2000, Bartel et al., 2011, Altizer, 2001, Altizer and Oberhauser, 1999) a known difference in immune response between males and female monarch butterflies (Lindsey and Altizer, 2009, Altizer and Oberhauser, 1999), which also have different dispersal tendencies (Bradley and Altizer, 2005), while in the European Corn Borer there was a differing effect of infection on the flight capabilities of the two sexes (Dorhout et al., 2011). This is investigated in more detail in Chapter 3.(Altizer and Oberhauser, 1999, Davis et al., 2005, Davis et al., 2012)

1.5.8 Determining the cost functions of infection in migratory species

Of the mechanisms described in sections 1.5.3 – 1.5.6, most the literature to date suggests that geographic disease dynamics are often too complex to be described by a single mechanism. For example, while disease levels may lead to migratory culling/ escape and an overall reduction in pathogen load in the monarch butterfly, migratory spread of the pathogen can still occur (Bartel et al., 2011). Alternatively, in the cane toad *Rhinella marina*, upregulation of immune components during periods of intense movement would be associated with migratory recovery (Brown and Shine, 2014), but this does not entirely counter-act the reductions in dispersal capacity caused by the lungworm *Rhabdias pseudosphaerocephala*, (Pizzatto and Shine, 2012).

In addition to presence of multiple mechanisms in a single system, the relevance of specific mechanism may also be affected by biotic and abiotic factors, for example age (Dorhout et al.,

2011), sex (Lindsey and Altizer, 2009) or environmental conditions (Roznik and Alford, 2015). In some cases, these biotic and abiotic factors can also be difficult to separate, for example when as seasonal changes are associated with variation in host immunity (Altizer et al., 2006).

A quantitative approach to understanding the impact of pathogen infection on migration which does not focus on the causal mechanism is disease cost functions. In a review proposed by Chapman et al. (2015b) the authors describe how multiple cost functions might exist between pathogen exposure and migratory capacity, as well as between migratory effort and disease susceptibility (see Figure 1.4). This approach addresses issues in the literature which cannot be entirely explained by causal mechanisms. For example, it describes how a step function could be used to explain why dispersal capacity in the monarch butterfly is only affected by protozoan infection when the disease load passes a certain threshold (Bradley and Altizer, 2005).

The approach of using disease cost functions is adopted in this thesis, but also adapted and expanded. In Chapter 3, the relationship between pathogen challenge and migratory capacity is explored in line with Figure 1.4a, but also expanded to include estimations of the cost functions associated with other aspects of the migratory phenotype such as weight and development time (Dingle, 2014). By quantifying how disease leads to physiological and behavioural changes associated with the overall migratory syndrome, this approach enables a more precise understanding of how migratory tolerance is affected by *Sf*MNPV infection in the fall armyworm.

In Chapter 4 the cost function between flight effort and pathogen load is explored, both in relation to time spent flying and the level of pathogen exposure, as per Figure 1.4b. This chapter also repeats the measures of disease tolerance recorded in chapter 3 (excellent science is built on repeatability and reproducibility after all, *especially* when results are contradictory) and uses this data to give insight into the migratory resistance in the *Sf*MNPV host-pathogen system. Finally, this these builds on the work by Chapman et al. (2015b) by assuming that multiple cost functions can exist in a single host pathogen system, and may be driven by physiological factors such as sex. (Dorhout et al., 2011, de Roode et al., 2008b)

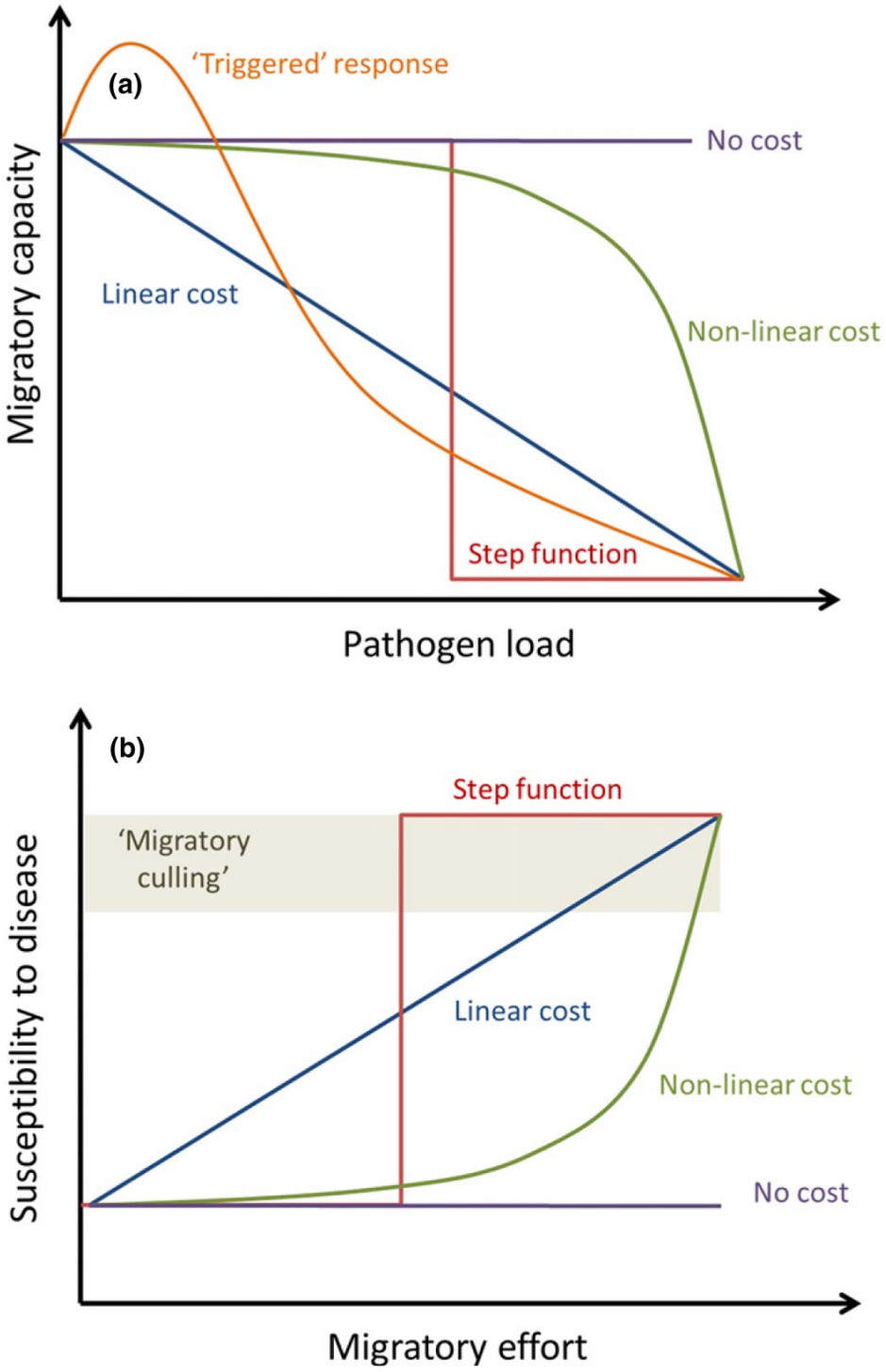

Figure 1.4: Proposed relationships between migration and disease, adapted from Chapman et al. (2015b). *As per author description:* "The interaction between migration and disease. (a) As pathogen loads increase, we might expect there to be a cost in terms of migratory capacity. This cost may be linear (blue line), nonlinear (green), a step function (red) or negligible (purple), depending on specific circumstances (see Box 1); low-level infections may also act as a cue triggering enhanced migratory capacity (orange). (b) Likewise, as migratory effort increases, so we expect a physiological cost, in terms of enhanced susceptibility to disease, and the shape of this cost function may also take a range of forms, with consequences for the evolution of migration and disease resistance. When costs exceed some threshold, then we can expect migration-induced susceptibility to infection to result in 'migratory culling' (shaded area), sensu Altizer et al. (2011); again, the point at which this threshold is reached will depend on the shape of the cost function."

1.6 PROJECT AIMS AND THESIS STRUCTURE

While there are multiple studies in the literature that have looked at the impact of disease on the migratory capacity of a host population and the different mechanisms that might drive the underlying migratory disease dynamics, this is still a very young field and there are few if any studies that explicitly seek to describe how standard theoretical concepts from the two different fields interact. Specifically, there has been no direct attempt to describe how the migratory syndrome (section 1.2.2) is affected by pathogen virulence or may alter depending on the level of host susceptibility (section 1.5.1). This is evident not only in instances where the dispersal of pathogens has been left out of the literature on animal movement (section 1.1) but also by the lack of studies which explicitly quantify changes in common disease traits such as susceptibility due to the effort invested in migration (Chapman et al., 2015b).

To address this, this thesis takes the framework from Chapman et al. (2015b) (Figure 1.4) and adapts it in order to quantify variation in the migratory tolerance and migratory resistance in an insect-baculovirus system. To this end, this thesis has four specific aims each of which is addressed in an individual chapter.

Chapter 2:

Lepidopteran migrants such as the fall armyworm lend themselves well to studies of migratory tolerance and migratory resistance. The rapid generation time allows the impact of infection to be quantified over the entire life history cycle of an individual. Their small size also makes them ideal candidates for rotational flight mill studies, which make it possible to capture and quantify the effort invested in flight to a level of detail that is not possible in birds and larger mammals. While rotational flight mills have been used for over sixty years, there are few studies that examine the extent to which they limit an individual's ability to fly or which of the multiple variables available are the most representative of the overall flight propensity. For this reason, rotational flight mills have predominantly been used to assess flight capacity rather than flight behaviour and there are no quantifiable studies to assess if the behaviour observed on rotational flight mills is comparable with the expectations of the definition of migration (section 1.1). As this thesis focuses on the impact of infection on the migratory syndrome, it was considered important to address these underlying issues in the methodology. As such, Chapter one aimed to:

1. Identify the most suitable variables for analysis in rotational flight studies.
2. Investigate the extent to which flight mills are a true reflection of the underlying drive to fly in a migratory insect.
3. Assess if the behaviour observed on rotational flight mills is comparable with the expectations of the definition of migration (section 1.1) across three noctuid species

4. Assess if the behaviour observed on rotational flight mills is comparable with the expectations of the definition of migration (section 1.1) within a species, where factors such as sex (Miller et al., 2011) and the upregulation of genes associated with the migratory phenotype (Jones et al., 2015) would be expected to correlate with changes in the migratory syndrome

Chapter 3:

Most studies to date that have considered the effect of infection in migratory species have focused on the implications for movement capacity rather than the migratory tolerance of disease. However, work such as that by (Schneider, 2011) would suggest that the impact of disease would differ depending on what stage in the infection cycle the host is attempting to migrate. There are also examples in the literature of where the impact of disease on migratory capacity is affected by the physiology of the host, including the reproductive status of migrating adults (Dorhout et al., 2011) and there seems to be strong evidence of Bateman's effect (differing immune responses between the sexes) altering the disease response in migratory species (Dorhout et al., 2011, Villacide and Corley, 2008) (see also section 1.5.7). This suggests that rather considering the effect of infection on migratory capacity in isolation, it is essential to understand the migratory tolerance of a host-pathogen system. To address this, Chapter 3 takes the approach described by Chapman et al. (2015b) to investigate the different cost functions of infection (Figure 1.4a, section 1.5.8) but adapts it with the aim of:

1. Quantifying disease cost functions across multiple life history traits (weight, development time and flight capacity) that are linked to the migratory syndrome to describe the effect of infection on migratory tolerance
2. Assess if variation in migratory tolerance within a species can be explained by Bateman's principle, which will lead to different cost functions in males and females.
3. Investigate the link between migratory tolerance and flight behaviour, and how this differs between the two sexes.

Chapter 4:

While Chapter 3 focuses on the effect of the pathogen infection on the migratory fitness of the host, Chapter 4 inverts this question and quantifies the effect of flight effort on pathogen load. With some notable exceptions, such as the diversity of influenza viruses in migratory birds (Li et al., 2014), much of the disease literature focuses on the fitness implications of infection on migratory hosts. However, even within this context, there are no studies that explicitly manipulate the distance travelled by a host to determine if, for a given level of infection, the

physical act of migrating reduces host fitness by reducing disease tolerance (Chapman et al., 2015b) or, conversely, if movement effort affects pathogen virulence. To address this, Chapter 4 uses the rotational flight mills, pathogen bioassays and molecular techniques to quantifying changes in pathogen load at different levels of flight effort. The specific aims of this chapter are to address the question of migratory resistance in the *Sf*MNPV system by:

1. Quantify the effect of flight on pathogen load as a function of
    a. The level of exposure to the pathogen
    b. The amount of time spent flying
2. Assess if there are differing migratory resistance function in the two sexes in line with Bateman's principle.
3. Assess the variability in the host-pathogen system by replicating the bioassay in Chapter 3 and determining the same cost functions for life history traits (weight and development time) that are linked to the migratory syndrome.

Chapter 5:

Having determined the effect of pathogen load on migratory tolerance and migratory resistance in Chapters 3 and 4, Chapter 5 uses a historic collection of insect samples from across the United States of America to investigate how results obtained in the laboratory relate to field observations. Specifically, chapter 5 aims to:

1. Quantify the level of seasonal and geographic variation in pathogen loads at 13 different sampling sites over a two-year period.
2. Assess how variation in the field relates to laboratory observations from chapters 3 and 4.

Chapter 6

Finally, Chapter 6 summarises all four chapters and discusses these results, both in relation to the level of variation observed in the system and within the broader context of migration-disease interactions.

CHAPTER 2.

INSIGHTS INTO INSECT FLIGHT BEHAVIOUR FROM ROTATIONAL FLIGHT MILLS

2.1 ABSTRACT

Understanding animal movement is a fundamental aspect of spatial ecology and population biology yet there are few detailed movement 'rules' that can be applied across multiple species. This is particularly true in insects, where technological limitations make it difficult to follow the movement pathway of a single individual over large distances. To try and gain an insight into the factors which affect insect flight, tethered flight is a commonly used laboratory practice but this has limitations and is used as a description of flight capacity rather than flight behaviour. This chapter assesses the extent to which rotational flight mills can be used to make inferences about migratory flight behaviour by comparing the flight parameters of three different species of migratory noctuid moth pests and comparing them to the standard definition of insect migration. By manipulating the weight of the central arm that insects have to turn while flying, heavier flight mills were shown to lead to a decrease in overall flight ability (speed, distance, duration) of the African Armyworm, *Spodoptera exempta,* which was the smallest of the three species studies. However, the number of flights for this species increased on heavier flight mills, suggesting these methods capture an underlying drive to fly. Investigations into the relationship between different flight parameters (speed, distance, duration and number of flights) found that in all three species, insects which flew the furthest distance did so by undertaking fewer, faster flights, demonstrating the persistent, straightened out flight behaviour associated with migration. An intra-species analysis shows that this persistent movement pattern was also associated with the upregulation of genes associated with the migratory syndrome in the cotton bollworm *Helicoverpa armigera* and supports sex-biased dispersal in the fall armyworm *Spodoptera frugiperda*. In combination, these studies have the potential to add to our fundamental understanding of the underlying nature of insect flight and how baseline relationships both within and across species follow very similar patterns which vary but do so consistently across different components that affect flight ability.

2.2 INTRODUCTION

Animal movement occurs on a wide variety of spatial and temporal scales. Understanding what drives this movement across taxa (Dingle, 2014a) is essential to gain an insight into large scale processes such as population dynamics (Clobert et al., 2009) and nutrient cycling (Bauer and Hoye, 2014). Animal movement, however, is formed of a complex suite of behaviours (Bauer and Klaassen, 2013) and defining this diversity is difficult, particularly when these definitions need to be integrated and understood across a wide range of taxa (Nathan et al., 2008) (Chapter 1).

In entomology, multiple technologies and research techniques exist that enable the study of insect movement at a local (Woodgate et al., 2016) or population scale (Chapman et al., 2011a) in the field, but the small size of insects means it is still not possible to follow the entire movement trajectory of a single individual over large distances (although see Wikelski et al., 2006). Thus, many studies use tethered flight studies conducted under laboratory and field conditions to gain an insight into insect flight behaviour (Chapter 1).

Tethered flight mills have been used in more than 100 studies (Appendix 1) to investigate the effects of a wide variety of physiological (Jones et al., 2016), environmental (Parker and Gatehouse, 1985a) and genetic (Jones et al., 2015, Parker and Gatehouse, 1985b) factors on insect flight capacity. Most of these studies use rotational flight mills (variations on Figure 2.1, Section 2.3.2) but these data are often used only as a proxy for flight capacity. Very little is known about how measurements obtained from rotational insect flight mills relate to behaviours such as migration, or if information from this system can give us insight into the underlying nature of insect flight.

Flight mill studies are usually comparative and rarely attempt absolute estimates of flight endurance. This is partly because very few studies have tried to understand how the flight mechanics on rotational flight mills may differ from those in free flight. One exception is (Riley et al., 1997), who estimated that *Cicadulina* leafhoppers required at least 20 – 30% of their mechanical power to overcome the friction and associated drag of rotational flight mills, and that the airspeed (the self-powered flight speed) of an insect would generate while flying on the mills was about a third of what they would attain in free flight. This suggests that flight mills under-estimate dispersal potential. However, it has also been observed that loss of tarsal contact can initiate (but not maintain) bouts of flight and this might lead to over estimates of flight propensity or behavioural differences (Edwards, 2006). Certainly, behaviour observed on rotational flight mills can differ to that observed under natural conditions or where insects are given the opportunity to respond to additional stimuli such as sex pheromones (Yamanaka et al., 2001). Overall however, data obtained from rotational flight mils has been shown to correlate closely

with dispersal capacity across a wide range of species (Jones et al., 2016) and such considerations may be less relevant in migratory insects, which are commonly assumed to show an inhibition of station keeping response when physiologically primed for migratory flight (Kennedy, 1985). For example, in the highly migratory African armyworm, which is known to have a pre-reproductive period during which mate-seeking responses are suppressed, flight periodicity was show to agree very well with that in field populations (Gatehouse and Hackett, 1980).

In addition to the technical complexities, analysis of rotational flight mill data has also proven difficult. Many studies introduce artificial cut-offs above which behaviour is defined as 'migratory' or 'dispersive' (Dingle, 1966, Bradley and Altizer, 2005), while other analyses focus only on single flight variables such as distance or duration (Elliott and Evenden, 2012, Cheng et al., 2012) and do not take into account factors such as periodicity or variation in behaviour over the flight period (see also appendix 1). Where this has been considered however, interesting insights into different flight strategies have been gained, such as Bruzzone et al. (2009) who used time-series analysis based on wavelets to distinguish three different behaviours (regular fliers, period fliers and pulsating fliers) which correlated closely with body mass in the woodwasp, *Sirex noctilio*

The aim of this chapter is three-fold. Firstly, this chapter aims to address the fundamental lack of information on how the flight mills limit insect flight capacity by comparing flight characteristics on two different sets of flight mills where the weight of the central arm varies between 1g and 3g. This study was undertaken in the smallest species, *Spodoptera exempta* to test the hypothesis:

i. The increase in effort required to turn the heavier rotational flight mills will lead to a decrease in all flight parameters (Riley et al., 1991);

The second aim of this chapter is to compare flight characteristics across three migratory species and determine if there is a common behaviour observed that supports the standard definition of insect migration (Kennedy, 1985) (Chapter 1, Section 1). The specific hypotheses tested are

ii. Increases in flight capacity (total distance flown) will be associated with flight behaviours that are in line with the persistent, straightened out movement definition of migration (Kennedy, 1985), specifically
    a. Insects which fly for longer will engage in fewer flights (Bruzzone et al., 2009);
    b. Insects with a longer flight duration would have to maintaining lower average flight speeds to sustain the increased flight effort;
    c. Insects which engaged in fewer flights will therefore fly at slower speeds;

The third and final aim of this chapter is to compare flight behaviour within species and assess if the characteristics associated with migration and defined in the second hypothesis (ii) are associated with sex-biased dispersal in the fall armyworm *Spodoptera frugiperda* and the upregulation of genes associated with the migratory phenotype in the cotton bollworm *Helicoverpa armigera.* For this I tested the following hypotheses:

iii. (Kennedy, 1985)Behaviour associated with migration (hypothesis ii, a - c) will be significantly increased in groups that have a higher migratory tendency, specifically;
    a. Females will show significant increases in migratory behaviour when compared to males, in line with expectations of sex-biased dispersal (Miller et al., 2011)
    b. Cotton bollworm populations from Dafeng will show significant increases in migratory behaviour when compared to populations from Anyang, as up-regulation of genes associated with the migratory phenotype were higher in this population (Jones et al., 2015)

Understanding how these behaviours are manifest on rotational flight mills is essential for data interpretation in Chapter 3, specifically where the effect of infection on migratory capacity is considered. However, it also gives credence to other key studies which have used rotational flight mills to investigate the effect of pathogen load on migratory capacity (Dorhout et al., 2011, Bradley and Altizer, 2005). Finally, demonstrating the specific way in which flight mills limit insect flight capacity (hypothesis i) helps to contextualise and validate the methods and results in Chapter 4.

2.3 METHODS

2.3.1 INSECT CULTURES

All three species of noctuid moths (*Helicoverpa armigera, Spodoptera frugiperda* and *S. exempta*) were maintained in the same insectary at 26 °C ± 2 °C, with a 16 h light: 8 h dark lighting regime. Insects were reared in individual 37ml pots lined with semi-artificial diet; an amended version of the wheat germ-based diet described by Smith (1998) for the two *Spodoptera* species and a chickpea-based diet for *H. armigera* (Grzywacz et al., 2011). Full rearing protocols are contained in Appendix 2.

Spodoptera exempta

To assess how the design, and the weight, of the flight mill arm affects flight behaviour, two different designs of the central arm were trialled (Fig. 2.1). The first weighs approximately 3 g excluding the bicolour disc. It has a double crossbar, incorporates a counter-balance and is made from galvanised iron with a diameter of 1.5 mm. The second design is much lighter, weighing approximately 1 g excluding the bicolour disc. The double cross bar and counter-balance have been removed from the previous version and the attachment mechanism simplified. The galvanised iron also has a much smaller diameter of 0.7 mm.

The African armyworm, *Spodoptera exempta*, is the smallest of the three available species, weighing approximately 180 mg, with a wing length of 14 mm – 15 mm (Aidley and Lubega, 1979). A total of 73 insects were used to assess differences between the two sets of mills; twenty-nine adult moths were flown on the 3 g mills for 6 consecutive nights and forty-four adults on 7 consecutive nights on the 1 g mills. Insects were imported from Lusaka, Zambia as pupae following a large-scale outbreak in December 2012. The culture was initially maintained at Lancaster University (25°C ± 2°C; 12h light:12 dark) and larvae from the first offspring generation (F1) were transported to Rothamsted Research as pupae. Larvae from the second generation (F2) reared through and the emergent adults used in the experimental study.

Spodoptera frugiperda

Investigations into the effect of baculovirus challenge on flight capacity in Chapter 3 showed a significant difference in flight capacity between the two sexes which had to be accounted for in addition to the impact of baculovirus challenge. Here I have extended that analysis to investigate how sex altered behavioural flight patterns.

A full description of the source population, treatments and rearing conditions is available in Chapter 3.

Helicoverpa armigera

Using the same flight mill system, Jones et al. (2015) demonstrated a significant difference in the total distance flown by different field populations of the cotton bollworm. Here I have reanalysed these data to investigate whether the different source populations exhibited changes in their flight patterns.

Larvae were collected in the summer of 2013 from Bt cotton at four separate field sites in eastern China: Dafeng, Jiangsu province; Anyang, Henan province; Jingzhou, Hubei province and Qiuxian, Hebei province. These were considered four separate field 'populations', although gene flow undoubtedly exists between at least some of the differing source populations. A full description of the methods can be found in Jones et al. (2015).

2.3.2 TETHERED FLIGHT MILLS

The rotational flight mills used in this study were developed at Rothamsted Research (Lim et al., 2013). An axis made from galvanised iron, the ends of which are sharpened to a fine point, is suspended from a central magnet (Figure 2.1 a). The flight mill arm extends outwards at a 90° angle from this axis, with the ends of the arm bent downwards at a 90° angle. The axis passes through the centre of a black and white bicolour disc (Figure 2.1 b), such that the disc rotates with the axis. As this disc passes the sensor (Figure 2.1 c), it is possible to measure the speed that the axis turns every five seconds, which enables measurements of distance that are accurate to within 10 cm. These data are captured in real-time by a micro-controller board and fed into a computer as the distance flown every five seconds. These raw data can then be converted into measurements of average and maximum speed, distance and duration of each flight as well as the time spent stationary between each flight. From these processed data, it is possible to extract 16 different aggregate flight variables (Table 2.1).

Each micro-controller board can accommodate up to eight flight mills. These are arranged into banks of 16 (Figure 2.1 d), with two micro-controllers in each bank. A total of forty-eight flight mills (three banks) are available.

Moths were attached to the flight mills using a galvanised iron handle with a base bent at 45° to the main axis. This is attached to the upper part of the thorax and the handle fits into a small piece of plastic tubing attached to the left-hand side of the flight mill arm (Figure 2.1 b) and, once attached, enables the moths to fly along a 50-cm circular trajectory.

To attach the handle to the insects, the moths were immobilised by either placing them on ice for five minutes (*S. exempta)* or in a cold room at 5°C - 7°C for 30 minutes to an hour (*S. frugiperda, H. armigera*). Once immobile, insects were placed on a piece of sponge and held in place with plastic mesh. The scales on the top of the thorax were gently removed with a cotton bud and a small amount of Evostick® Impact Instance Contact Adhesive applied to both the thorax and the base of the flight mill handle. The glue was left to dry for five minutes before attaching the handle, after which the insects were returned to the cold room to keep them immobile. This was necessary to stop them from damaging their wings and detaching the flight mill pins.

All insects used in this study were flown within 12 – 24 hours of eclosion. Two hours before being placed on the flight mills insects were fed a honey solution. Insects that refused to feed voluntarily were encouraged to feed by unravelling the proboscis and placing it on the cotton wool soaked in honey solution (Figure 2.1 f).

Moths were attached to the mills at a range of times between 18:00 and 19:30, prior to lights off at 20:00. In the absence of tarsal contact, moths have a reflexive tendency to fly. To limit this and get an accurate estimate of first flight time, insects were given a 2cm x 2cm piece of paper (Figure 2.1 b) that provided the necessary tarsal contact. The insects were left to fly over night and taken off the mills between 09:00 and 10:00 am.

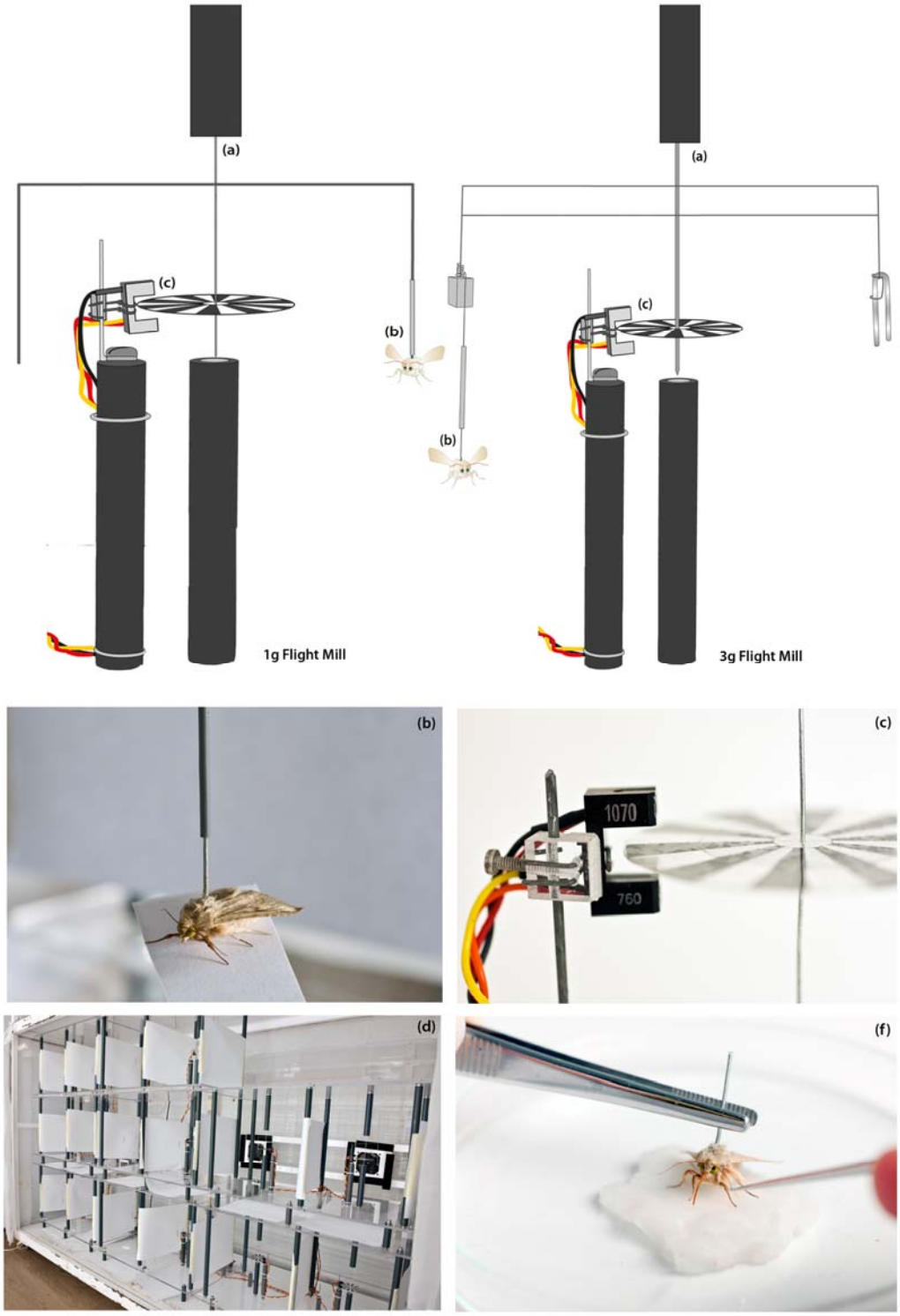

Figure 2.1: Illustration of the tethered flight mills. The design of the central flight mill arm was adapted so that the weight was reduced from 3 g (top right) to 1 g (top left). The flight mills work by suspending a central axis, the top of which is sharpened to a fine point, from a magnet (a). Insects are attached to the fight mill arm by gluing a galvanised steel handle to the thorax and inserting the handle into a piece of plastic piping which is suspended from the flight mill arm (b). As the moth flies, it rotates around a 50 cm circular trajectory. A bicolour disc that is suspended from the central arm turns with the axis as the moth flies and passes through a light sensor (c). This data is passed to a micro-controller board, which allows the recording of the distance flown every five seconds and is accurate to within 10 cm. Each micro-controller board can accommodate up to eight flight mills. These are arranged into banks of sixteen (d), with two micro-controllers in each bank. Prior to flight, moths are fed a honey solution. Those moths which refuse to feed can be forced by extending the proboscis (e).

2.3.3 Statistical Analysis

Principal Components Analysis

The basic flight mill data (distance flown every five seconds) can be manipulated to extract a wide variety of different behavioural response variables. In this study, I extracted 16 different variables (Table 2.1) and assessed the levels of covariation between them using principal components analysis (PCA).

Principal components analysis is a form of multidimensional scaling that reduces a large number of highly correlated variables into a small number of uncorrelated principal components. Each principal component is formed of a linear combination of the original variables called loadings, which describe the contribution of each variable to the principal component. As such, rather than reflecting the measurements in the original data such as speed or distance, principal components describe the variation in these data.

Where the response variables are highly correlated (as is the case with the variables in Table 2.1, R^2 values in the range of 0.3 - 0.9) the first two principal components are often enough to account for most the variation in the dataset.

By plotting the loadings of each original variable against the first two principal components (Fig 2.2), it is possible to show relationships which may not be apparent in the original multi-dimensional dataset and identify explanatory variables for further investigation. A full description of the mathematical rationale underpinning PCA can be found in Manly (1994).

Principal components analysis does not require the data to be normally distributed, but some variables were transformed to reduce the influence of outliers (Table 2.1).

	Abbreviation	Variable	Description	Transformation
Distance parameters	**TDist_sqrt**	**Total Distance Flown (m)**	**Sum of the distance of all individual flights**	**Square root**
	AvDist_log10	Average Flight Distance (m)	Mean flight distance	Log 10
	Furthest.Dist.log10	Distance of Furthest Flight (m)	The flight which achieved the greatest distance	Log 10
	Furthest.Dur.log10	Duration of Furthest Flight (s)	The duration of the flight which achieved the greatest distance	Log 10
	Furthest.MS	Maximum Speed of Furthest Flight (m/s)	The maximum speed attained in the flight that achieved the greatest distance	-
Duration parameters	Tdur.sqrt	Total Flight Duration (s)	Sum of the duration of all individual flights	Square root
	AvDur.log10	**Average Flight Duration (s)**	**Mean flight duration**	**Log 10**
	Longest.Dur.log10	Duration of Longest Flight (s)	The flight with the longest duration	Log 10
	Longest.Dist.log10	Distance of Longest Flight (m)	The distance covered in the flight with the longest duration	Log 10
	Longest.MS	Maximum Speed of Longest Flight (m/s)	The maximum speed attained in the flight with the longest duration	-
Speed parameters	**MaxFSpeed**	**Maximum Flight Speed (m/s)**	**Maximum flight speed attained**	**-**
	AvSpeed	**Average Flight Speed (m)**	**The mean flight speed, based on the mean speed of each individual flight**	**-**
	Fastest.Dist.log10	Distance of Fastest Flight (m)	Distance flown in the flight with the fastest maximum speed	Log 10
	Fastest.Dur.log10	Duration of Fastest Flight (s)	Duration of the flight with the fastest maximum speed	Log 10
Other	**NumFlights.log10**	**Number of Flights**	**The number of flights recorded**	**Log 10**
	Prop1.sqrt	Proportion of flights > 1000 seconds	The proportion of flights with a duration of more than 1000s (16.67 minutes)	Square root

Table 2.1: Variables used in principal components analysis (PCA) The rotational flight mills record the distance flown every five seconds, and from this raw data it is possible to calculate a wide variety of different variables that summarise flight behaviour. In this study, sixteen different variables were extracted and compared using principal components analysis (PCA). Principal components analysis showed variables clustering into three distinct categories (Figure 2.2): Distance/ duration, speed and number of flights. Variables highlighted in bold were selected from these three categories for further detailed analysis. Where necessary, variables were transformed to reduce the influence of outliers which might unduly affect the PCA. These transformed variables were also used in further analysis.

Quantifying relationships between flight variables to aid behavioural interpretation of flight mill data

For all three species, the following relationships were investigated:

1. Total distance flown (TDist) (m) as a function of number of flights (NumF)
2. Average flight duration (AvDur) (s) as a function of number of flights
3. Average flight duration (s) as a function of average speed (AvSpeed) (m/s)
4. Number of flights as a function of average speed (m/s)
5. The duration of every flight undertaken by each individual (IndvDur) (s) as a function of the average speed for every flight undertaken by an individual moth (IndvAS) (m/s)
6. The duration of every flight undertaken by each individual (s) as a function of the maximum speed for every flight undertaken by an individual moth (IndvMS) (m/s)

For all three species, mixed effects models were used. These quantified the interaction between the six flight variables listed above and any relevant "influencing" factors (arm weight is *S. exempta,* source population is *H. armigera* and sex in *S. frugiperda*). They also accounted for the effect of pupal weight (Wilson and Gatehouse, 1992). The starting model used was:

$$\textit{Flight variable 1} = \alpha + \beta_1 \times \textit{pupal weight} + \beta_2 \times \textit{Flight variable 2} + \beta_3 \times \textit{influencing factor} + \beta_4 \times \textit{Flight variable} \times \textit{influencing factor}$$

To ensure that the relationship between individual flights and maximum/average speed was not a result of resistance on the flight mills, all flights lasting less than 30 seconds were excluded from analysis.

The design of these experiments made it necessary to account for a variety of random terms (i.e. terms that may account for variation in the data but are not of biological interest in themselves): the day on which insects were flown (all three species), flight mill set up (all three species) and sibling group (not recorded for *Helicoverpa armigera*). To assess if these accounted for a significant amount of variation, each term was fitted as a random intercept and compared to an ordinary linear regression model using a likelihood ratio test, where significance estimates were obtained by testing whether the variation explained was greater than zero ($\beta > 0$). This follows a Chi-square distribution with p degrees of freedom (χ_p^2), where p is obtained using a likelihood ratio test (Zuur et al., 2009). Where all three terms were not significant ($p > 0.05$), random terms were removed from further analysis and the starting model investigated using a standard linear model (LM). Where one or more random terms accounted for a significant amount of variability ($p < 0.05$), linear mixed effects (LME) models were implemented. For models where individual flights were the response, flights were pseudo-replicated by individual and this term was included

in LME models by default. Terms were dropped sequentially from the full model using either F tests (linear models) or conditional F tests (LME models). Where necessary, both response and explanatory variables were transformed to meet the requirements of normality (Table 2.1). The assumptions of normality, heterogeneity and influential observations were assessed by plotting the residuals.

The effect of sex, source population and flight mill design on flight capacity

To assist with the interpretation of the above relationships, it was necessary to investigate the effect of geographic source population (*H. armigera*) and arm weight (*S. exempta*) on overall flight capacity. Four flight variables were selected from the PCA output and investigated: total distance flown, average flight duration, maximum speed and number of flights. Random factors were tested as above but did not contribute to a significant proportion of the variation ($p > 0.05$). The effect of the weight of the flight mill arm on flight capacity in *S. exempta* was investigated using t-tests. In *H. armigera*, the following full model was assessed using the same LM methodology above:

$$Flight\ variable = \alpha + \beta_1\ x\ pupal\ weight + \beta_2\ x\ source\ population$$

The effect of sex on the four flight variables in *S. frugiperda* is given in Chapter 3 and Appendix 4.

Software

All analysis was carried out using R version 3.3.0 "Supposedly Educational" (R Core Team, 2016). Linear models and PCA were carried out using the function *princomp*() and *lm*() functions in the base R package. Linear mixed effects models were implemented in *LME4* (Bates et al., 2014) with p-values derived from *lmerTest* (Kuznetsova et al., 2016). Restricted Maximum Likelihood (REML) assessments of random terms were carried out using the library *nlme* (Pinheiro J et al., 2014).

2.4 RESULTS:

2.4.1 PRINCIPAL COMPONENTS ANALYSIS

The total variation for the first two principal components (PC1 and PC2) described large amounts of variation for all three species: 83.0% for *S. exempta*, 89.2% for *H. armigera* and 82.5% for *S. frugiperda.*

Biplots of loading contributions for individual flight variables against the first two principal components showed covariation of flight variables clustering into three distinct categories for all three species: speed, distance/duration, and number of flights (Figure 2.2, Figure 2.3 and Figure 2.4). Using this information, the following flight variables were chosen for further analysis: total flight distance (m), average flight duration (s), number of flights, and maximum and average flight speed (m/s) (Table 2.1).

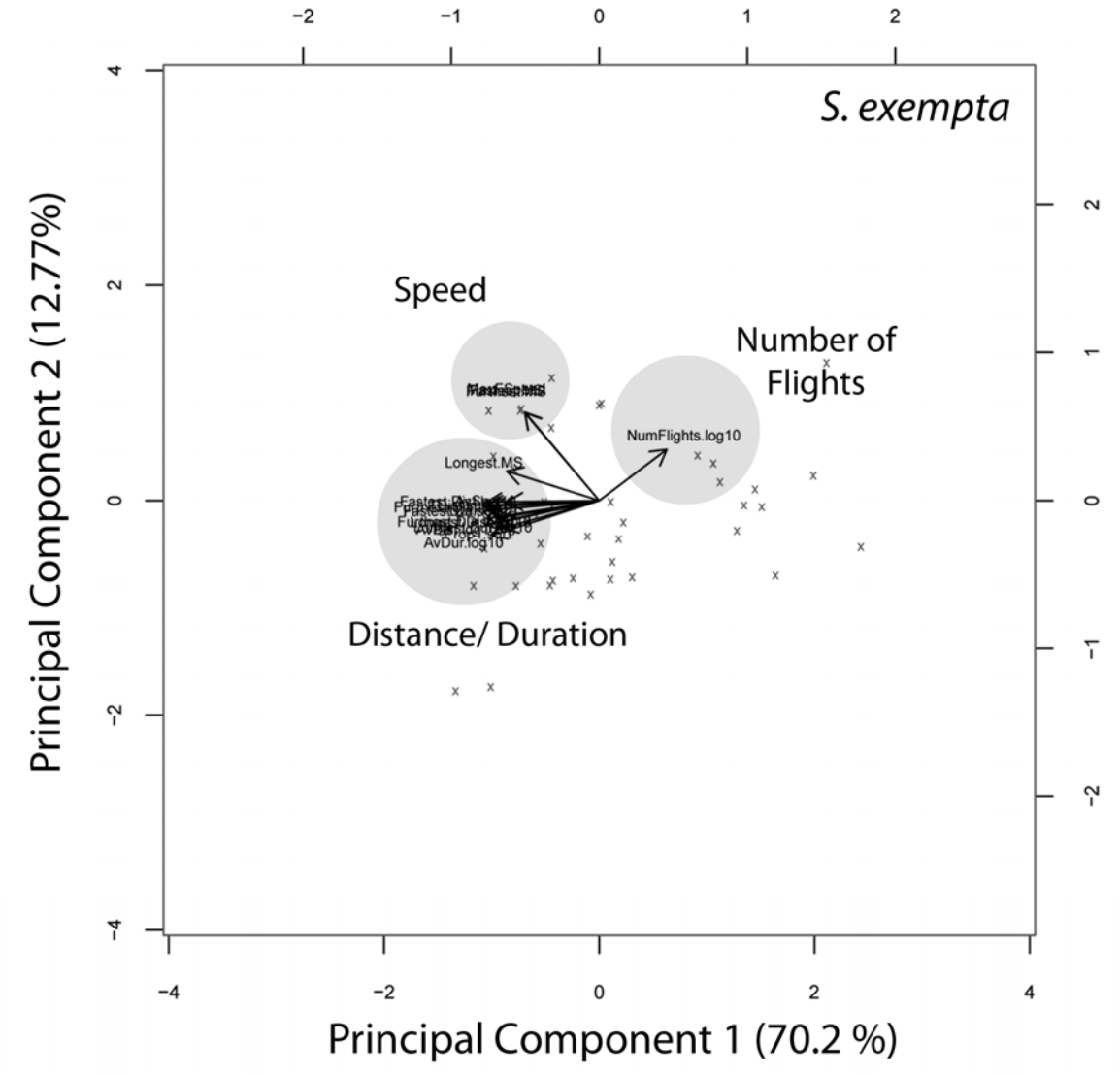

Figure 2.2: Results of the principal components analysis (PCA) for *Spodoptera exempta.* These results show the flight behaviours in Table 2.1 clustering in three distinct categories: speed, distance/duration and number of flights. The x and y axes show the variation captured by the first two principal components (PC1 and PC2). The score for each insect is shown with 'x' (n = 44). The proportional contribution of each loading is equivalent to the length of the arrow. Similar results are shown for *H. armigera* and *S. frugiperda*.

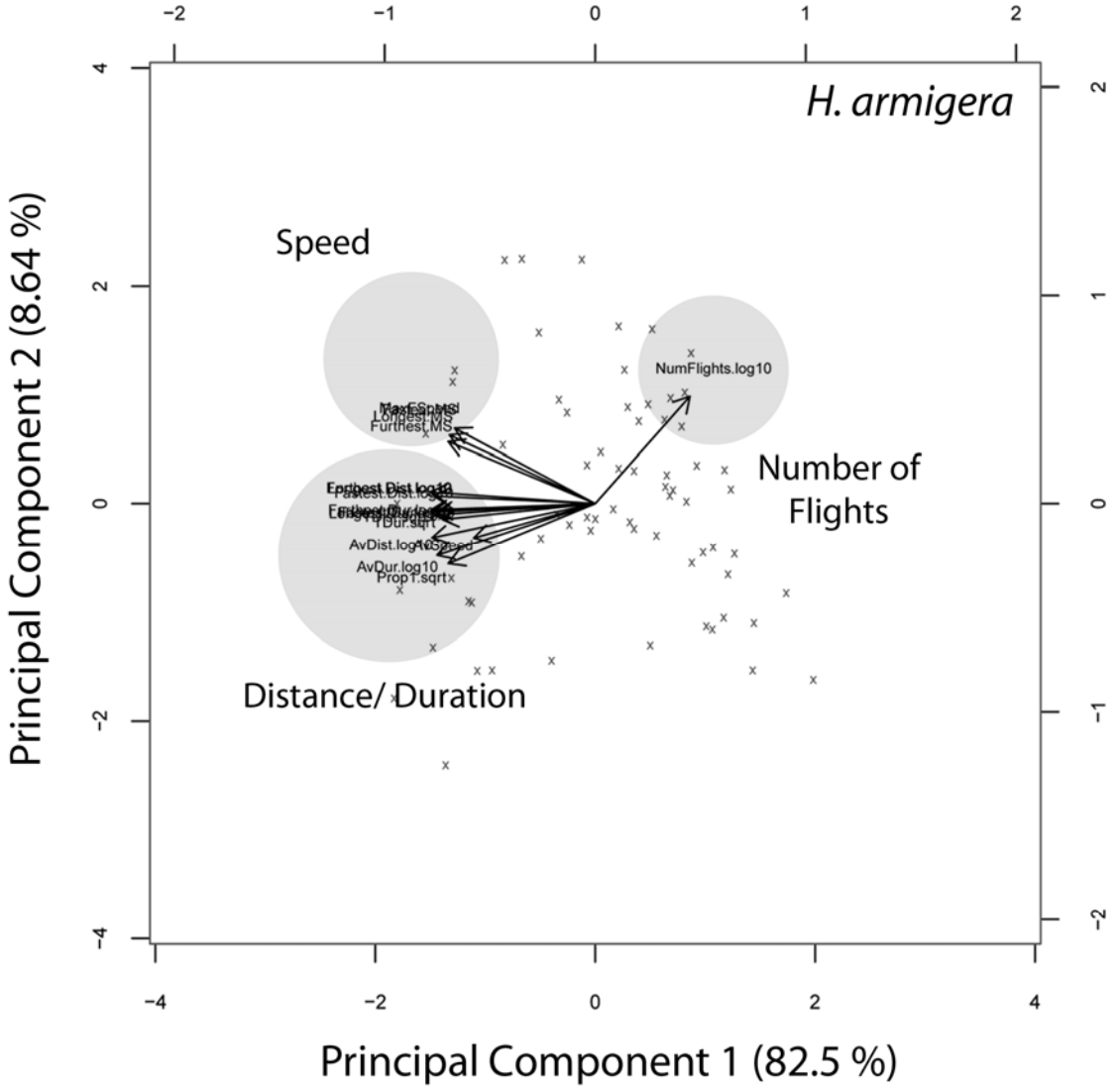

Figure 2.3: Results of the principal components analysis for *Helicoverpa armigera.* As with *S. exempta* and *S. frugiperda* the output of the PCA shows the flight behaviours in Table 2.1 clustering in three categories: speed, distance/duration and number of flights. The x and y axes show the variation captured by the first two principal components (PC1 and PC2). The score for each insect is shown with 'x' (n = 73 from 4 geographic locations). The proportional contribution of each loading is equivalent to the length of the arrow.

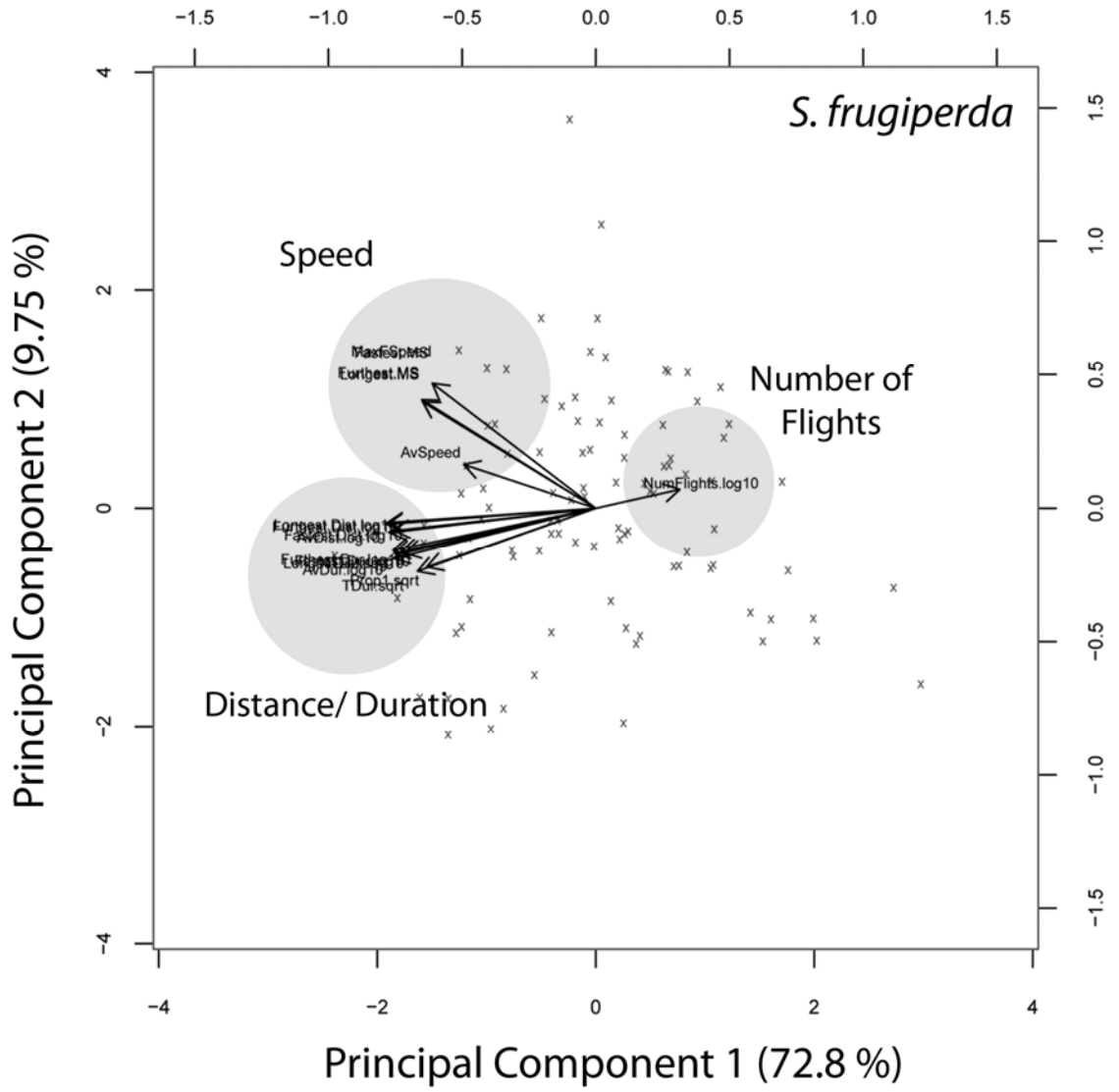

Figure 2.4: Results of the principal components analysis for *Spodoptera frugiperda.* The results from the PCA analysis, demonstrating that the flight behaviours in Table 2.1 clustering into three categories: speed, distance/duration and number of flights. The x and y axes show the variation captured by the first two principal components (PC1 and PC2). The score for each insect is shown with 'x' (n =99, 55 females and 44 males). The proportional contribution of each loading is equivalent to the length of the arrow. Similar results are shown for *S. exempta* and *H. armigera*.

2.4.2 THE EFFECT OF SEX, SOURCE POPULATION AND FLIGHT MILL DESIGN ON FLIGHT CAPACITY

Spodoptera exempta

Insects flown on the lighter flight mills flew faster (t-test: $t_{68.28} = 6.1$, $p < 0.001$, Figure 2.5c) and for longer (t-test: $t_{53.08} = 4.15$, $p < 0.001$ Figure 2.5b), achieving a greater total distance (t-test: $t_{64.11} = 4.02$, $p < 0.001$, Figure 2.5 a). Number of flights, however, was higher on the heavier 3 g mills (t-test: $t_{59} = -3.81$, $p < 0.001$, Figure 2.5d)

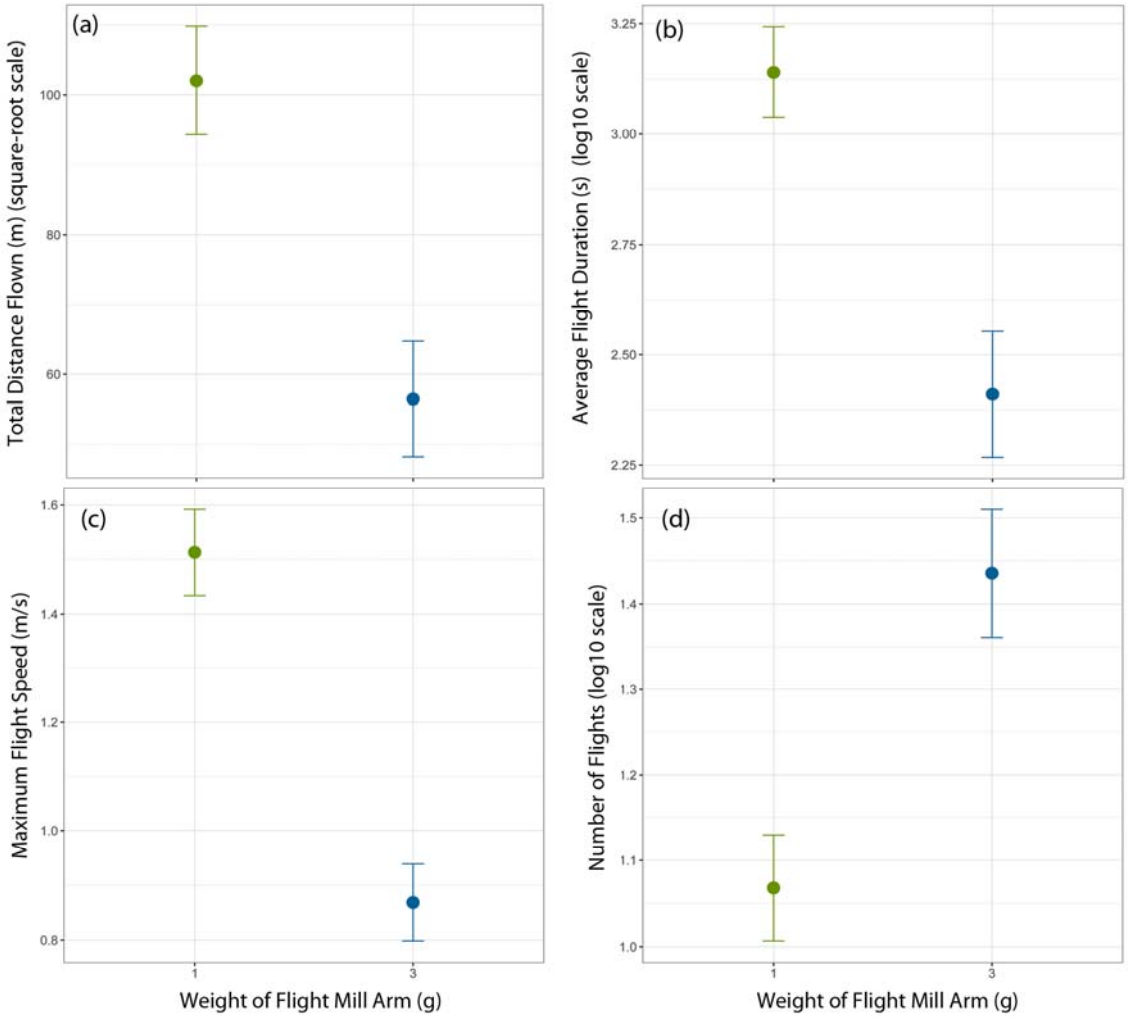

Figure 2.5 The effect of the weight of flight mill design on flight capacity in *Spodoptera exempta*. The smallest of the three species, *Spodoptera exempta,* was flown on two separate designs on the flight mills (Figure 2.1) which weighed 1g and 3g respectively. Insects on the lighter flight mills flew for significantly greater duration at significantly faster speeds, achieving greater distances over all. Number of flights however was higher on the heavier mills suggesting that, although insects were limited in their capacity to turn the flight mills, the flight mills were still capturing an underlying behavioural drive to fly.

Helicoverpa armigera

There were significant differences in total distance flown (LM: $F_{3,39} = 4.09$, $p = 0.009$, Figure 2.6a), average flight duration (LM: $F_{3,39} = 3.86$, $p = 0.01$, Figure 2.6 b) and number of flights (LM: $F_{3,39} = 3.00$, $p = 0.04$, Figure 2.6 c) for two of the four source populations, with insects from Dafeng flying significantly longer, achieving greater total distances and engaging in fewer flights than those from Anyang. There was no effect of source population on maximum flight speed ($p > 0.05$). These findings are in line with those in Jones et al. (2015)

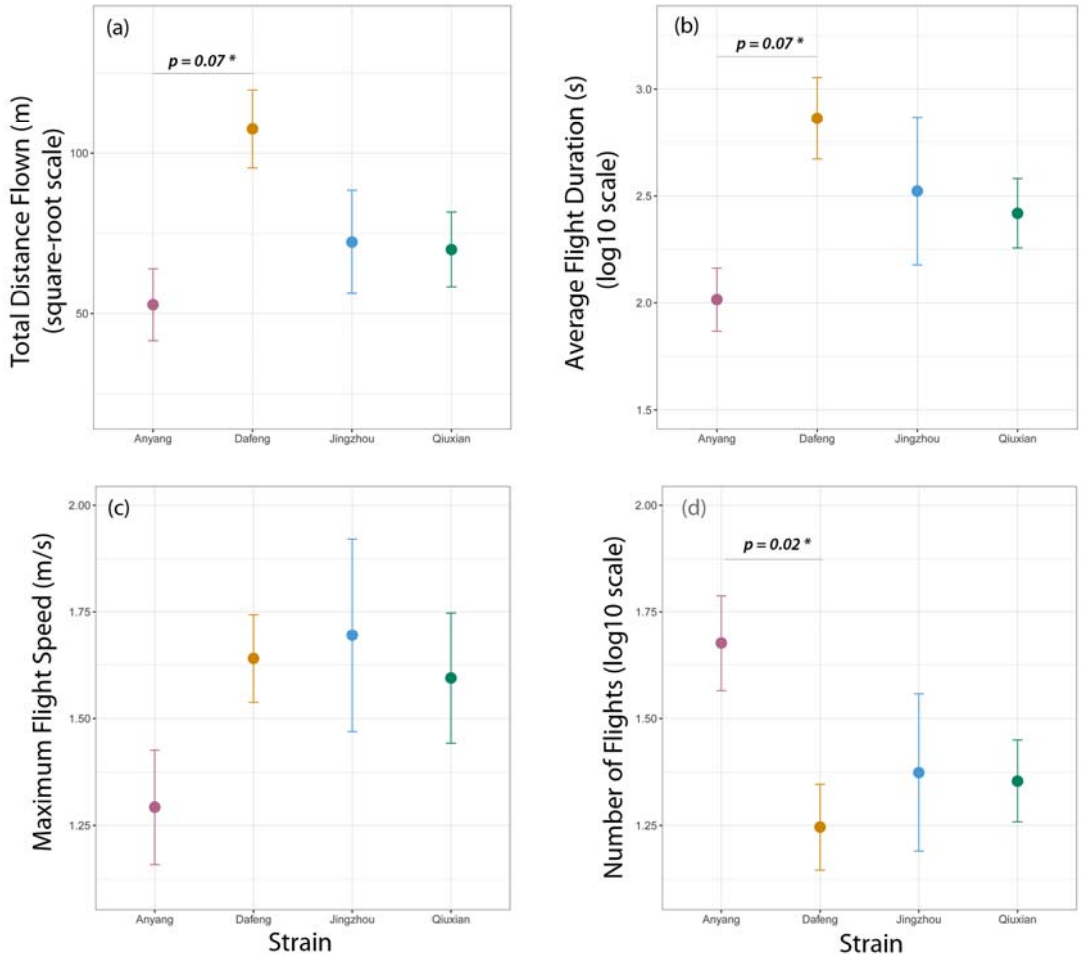

Figure 2.6: Differences in flight capacity for geographic strains of *Helicoverpa armigera*. Insects from Dafeng (Jiangsu province) flew for significantly longer and undertook significantly fewer flights than insects from Anyang (Henan province), achieving significantly larger total distances. As insects from Dafeng also showed a significant increase in the up-regulation of genes associated with the migratory syndrome (Jones et al., 2015) this suggests the behaviours observed are in line with the expectations of migratory flight. There was no significant difference in speed for any of the four geographic strains and insects from Jingzhou and Qiuxian could not be statistically distinguished from any other strain. See text for significance levels.

Spodoptera frugiperda

Full results for the differences in total distance flown, average flight duration, maximum speed and number of flights between the two sexes can be found in Chapter 3, in which females engaged in more flights of longer average duration than males and hence achieved greater total distances.

2.4.3 QUANTIFYING RELATIONSHIPS BETWEEN FLIGHT VARIABLES TO AID BEHAVIOURAL INTERPRETATION OF FLIGHT MILL DATA

For all three species, insects that flew for longer periods of time and achieved greater distances, engaged in fewer flights. Insects with fewer flights also flew faster. Flight duration was positively correlated with flight speed. The nature of these relationships, however, depended on the influencing factor (Figure 2.7, Figure 2.9 and Figure 2.11), discussed for each species below. There was no effect of pupal weight on flight behaviour for the two species where these data were available (*H. armigera* and *S. frugiperda*) ($p > 0.05$).

Spodoptera exempta

The variable *Number of flights* included three influential observations: two insects that flew fewer than 3 times, and one that engaged in more than 176 flights (<0.5 and >2.25 on the log10 scale). As there was no obvious justification for removing these values, the dataset was analysed both with and without them.

If the influential observations were excluded, total distance flown (LM: $F_{1,66} = 14.32$, $p < 0.001$, Figure 2.7a) and average flight duration (LM: $F_{1,66} = 50.38$, $p < 0.001$, Figure 2.7 b) correlated negatively with number of flights. The number of flights also correlated negatively with average flight speed (LM: $F_{2,65} = 29.95$, $p < 0.001$, Figure 2.7c), with a significantly different intercept for each of the two sets of flight mills, such that insects on the heavier mills, which achieved the same speed as insects on the lighter flight mills, engaged in more flights on average (LM: $F_{2,65} = 8.09$, $p = 0.005$, Figure 2.7c).

If the influential observations were included, the underlying negative relationship between total distance/average duration and number of flights remained the same (LM: *TDist*: $F_{2,69} = 16.12$, $p < 0.001$; *AvDur*: $F_{3,68} = 31.39$, $p < 0.001$). This was also true for the negative relationship between number of flights and average speed (LM: $F_{1,70} = 31.02$, $p < 0.001$). However, these observations did affect the significance of the slope and intercepts in the models. For the relationship between number of flights and average speed, the intercept for the different designs was no longer significant ($p > 0.05$). There was, however, a significant difference in intercept for total distance flown, with insects on the lighter mills flying further (LM: $F_{2,69} = 3.03$, $p = 0.016$). There was also a significant interaction between average flight duration and number of flights, with moths on the lighter flight mills engaging in fewer flights that lasted longer (LM, log10 scale: $F_{3,68} = 4.13$, $p = 0.046$ *1g mills:* $\beta = -1.24 \pm 0.22$, $t_{42} = -5.68$, $p < 0.001$; 3*g mills:* $\beta = -0.15 \pm 0.29$, $t = -1.8$, $df = 26$, $p = 0.084$). As these slopes and interactions were influenced by only three observations, influential observations are excluded from Figure 2.7 but full parameter estimates of the data can be found in Appendix 3.

For the relationship between average flight duration and average flight speed, there was a significant interaction showing that insects which flew for longer achieved greater speeds, but the speed achieved on the heavier mills was significantly slower for the equivalent flight duration (LM: log10 (*AvDur*) ~ *AS*: $F_{3,68} = 8.54$, p = 0.005, *1 g mills:* β = 2.50 ± 0.39, *3 g mills:* β = 5.48 ± 0.94, not shown).

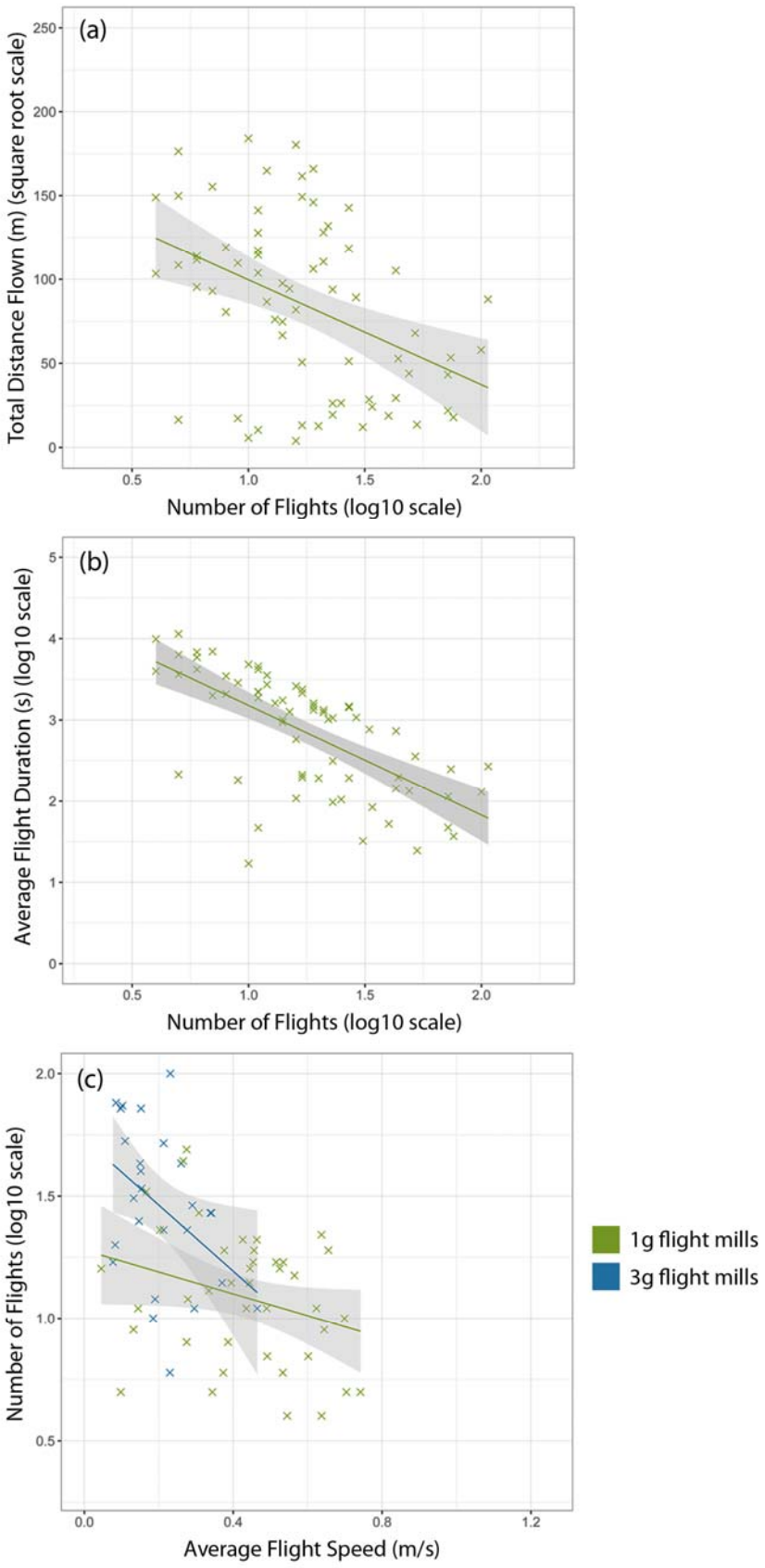

Figure 2.7: The relationship between flight variables for *S. exempta*. Significant negative relationships between total distance and number of flights (a), average flight duration and number of flights (b) and number of flights and average speed (c) suggest that insects which fly further do so by engaging in fewer flights. Those flights are also faster in insects that flew further. This is in line with the standard definition of migration (Kennedy, 1985) and is comparable to what was observed in the other noctuid species studied. The weight of the flight mill arm was only found to significantly affect the relationship in (c), with insects on lighter mills attaining greater speeds. At lower speeds, insects on the heavier mills undertook more flights for equivalent speeds. Grey bands show 95% confidence intervals. Full statistics are given in the text.

For every flight undertaken by every individual, longer flights again resulted in increased flight speeds, with an interaction between speed and the weight of the flight mill arm. This relationship was such that insects on the lighter mills achieved greater flight speeds for equivalent durations (LME for *maximum flight speed* correcting for pseudo-replication by individual: $t = 2.24$, $n = 1,273$, $p = 0.03$, *1 g mills:* $\beta = 1.05 \pm 0.05$, *3 g mills:* $\beta = 1.75 \pm 0.18$, Figure 2.8; LME for *average flight speed* correcting for pseudo-replication by individual: $t = 2.77$, $n = 1,273$, $p = 0.006$ *1 g mills:* $\beta = 1.16 \pm 0.12$, *3 g mills:* $\beta = 1.27 \pm 0.08$).

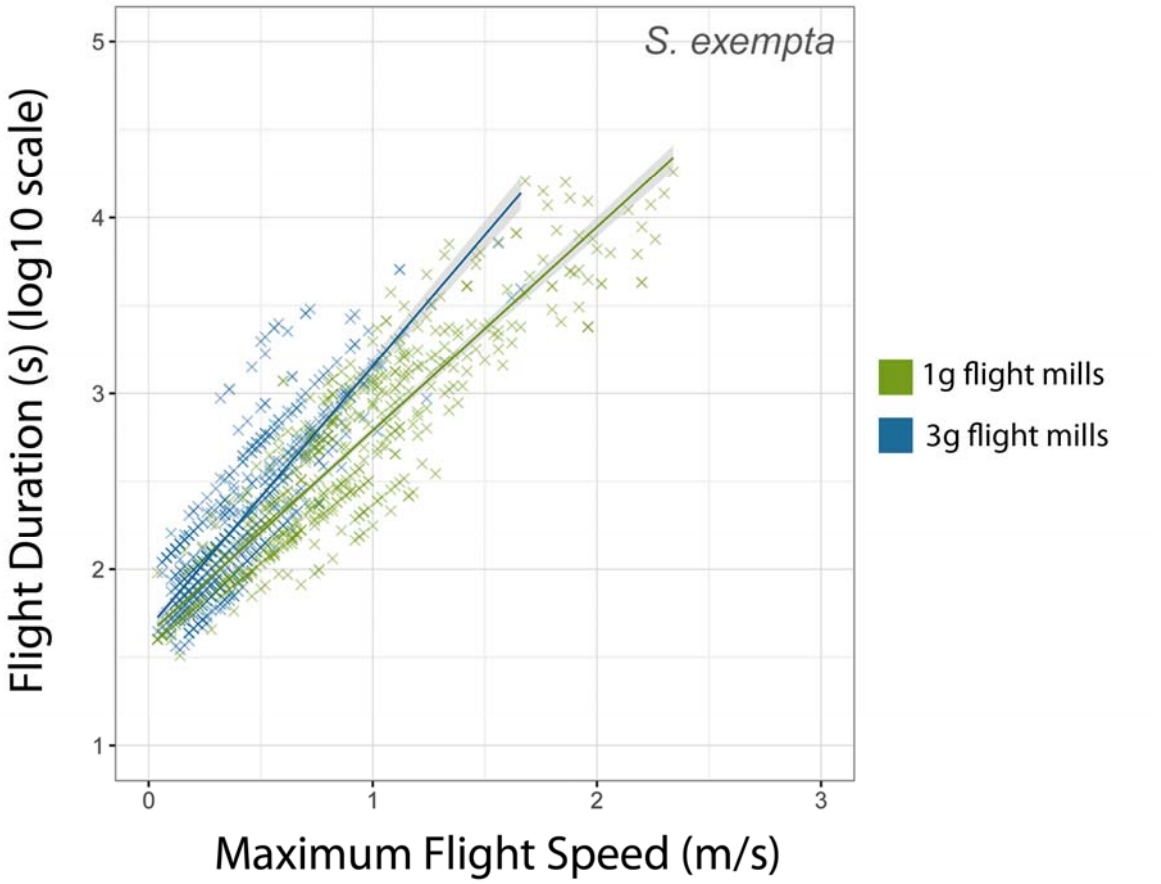

Figure 2.8: The relationship between flight speed and duration for every flight undertaken by every individual in *S. exempta*. The longer a flight lasted, the faster that flight was on average, in line with the standard definition of migration (Kennedy, 1985). There was also a significant interaction between speed and the weight of the flight mill arm, such that on the heavier mills there was a reduced speed for the equivalent flight duration. Significance levels can be found in the text and full model parameters are available in Appendix 3.

Helicoverpa armigera

In the cotton bollworm, source population did not introduce any significantly different intercepts or interactions ($p > 0.05$) for the averaged flight variables. Increases in total distance flown and average flight duration resulted in a reduction in the number of flights (LM: *TDist:* $F_{1,71} = 28.11$, $p < 0.001$; LM: *AvDur:* $F_{1,71}=104.81$, $p < 0.001$; Figure 2.9 a, b) and increased number of flights resulted in a significantly slower average flight speed (LM: $F_{1,71} = 27.32$, $p <0.001$, Figure 2.9 c). As in the other species, average flight duration was positively correlated with average flight speed (LM: $F_{1,71} = 94.59$, $p < 0.001$).

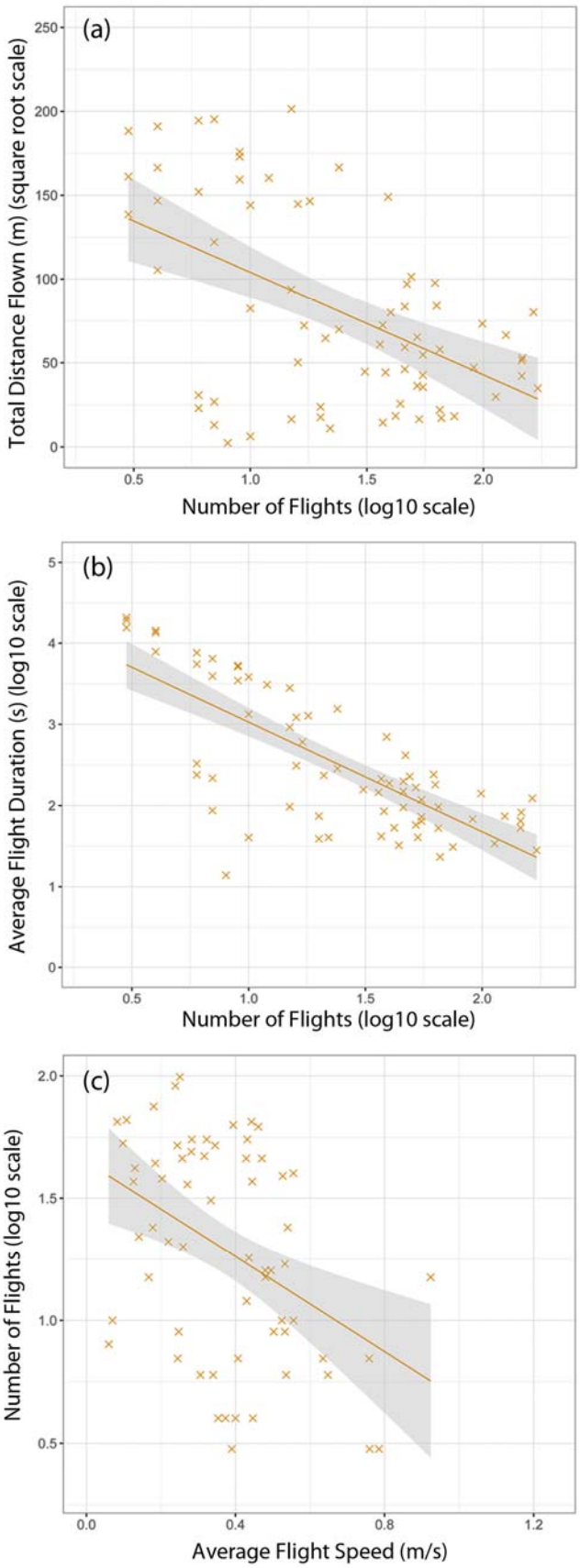

Figure 2.9: the relationship between flight variables for ***H. armigera***. As with both S. *exempta* and *S. frugiperda*, negative relationships were observed between total distance flown and number of flights (a), average flight duration and number of flights (b) and number of flights and average flight speed (c). This supports the Kennedy (1985) definition of migration, as insects which flew the furthest did so by engaging in fewer, faster flights. Grey bands show 95% confidence intervals. Full statistics can be found in the text.

For individual flights, longer flights resulted in increased flight speeds. This is the same relationship found in the other two species. There was also a significant interaction between flight speed and source population (LME for *maximum flight speed* correcting for pseudo-replication by individual: $F = 23.35$, $n = 1{,}476$, $p < 0.001$, *Anyang:* $\beta = 0.54 \pm 0.04$; *Dafeng:* $\beta = 1.05 \pm 0.01$, *Jingzhou:* $\beta = 0.86 \pm 0.07$, *Qiuxian*: $\beta = 0.86 \pm 0.04$,Figure 2.10; LME for *average flight speed* correcting for pseudo-replication by individual: $F = 15.49$, $n = 1{,}476$, $p < 0.001$, *Anyang:* $\beta = 0.91 \pm 0.09$; *Dafeng:* $\beta = 1.76 \pm 0.10$, *Jingzhou:* $\beta = 1.35 \pm 0.13$, *Qiuxian*: $\beta = 1.48 \pm 0.09$).

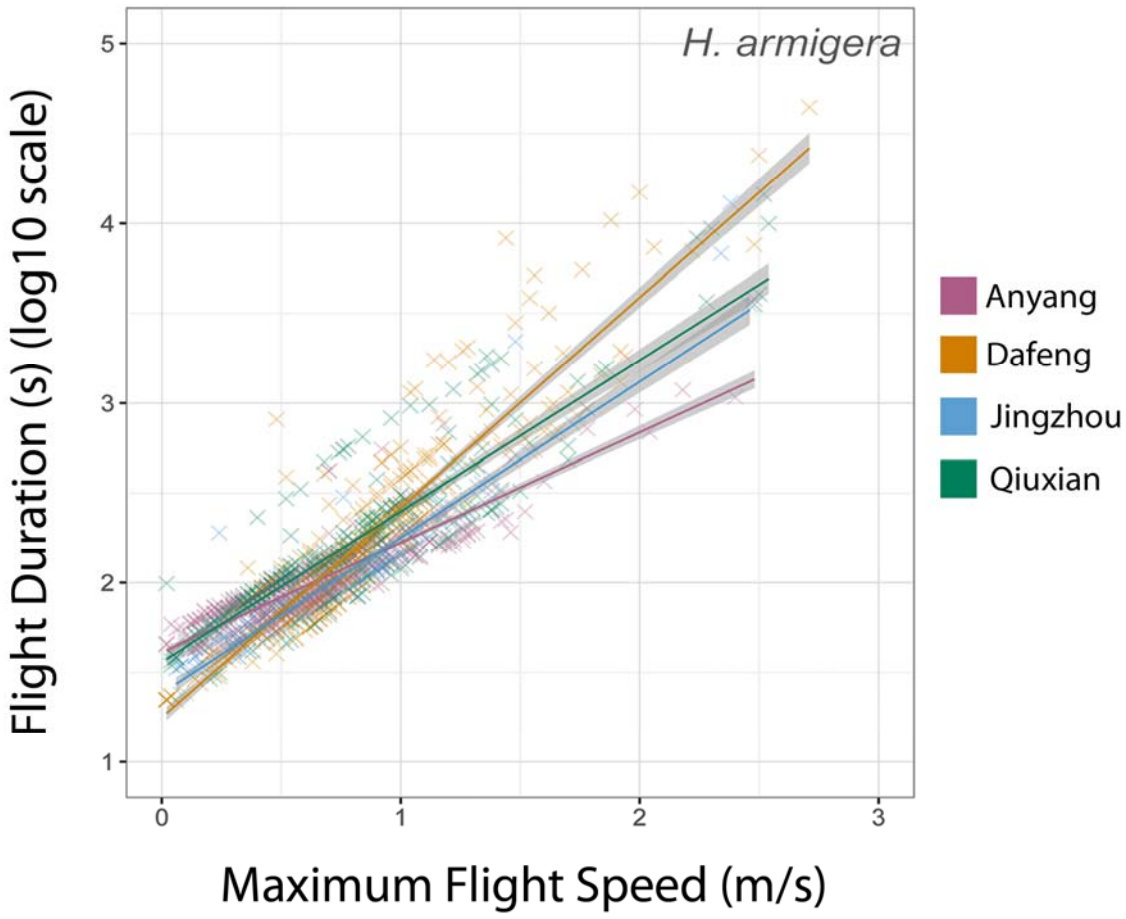

Figure 2.10: The relationship between flight speed and duration for every flight undertaken by every individual in *H. armigera*. The significant interaction between strain and flight speed shows flights of an equivalent speed lasted longer on average in populations where there was an up-regulation of the genes associated with the migratory syndrome (Daeng) than in populations where there were reduced levels of expression (Anyang). Grey bands show 95% confidence intervals. Significance levels can be found in the text and full model parameters are available in Appendix 3.

Spodoptera frugiperda

Total distance flown correlated negatively with number of flights for females but was not significant for males (LM: t_{42}=0.26, p = 0.80, $F_{3, 95}$ = 7.45, p = 0.008, *females:* β = -58.10 ± 14.09, t_{53} = -4.12, p < 0.001; *males:* β = 4.79 ± 18.21, Figure 2.11 a). Average flight duration correlated negatively with number of flights and there was a significant interaction between the two sexes which demonstrates that, where the two sexes undertook the same number of flights, those flights were longer on average in females, but only where the number of flights was relatively low. Where the number of flights was above several hundred, there was no longer a difference between the two sexes (LM: f*emales:* β = -1.36 ± 0.18, t_{53} = -7.643, p < 0.001; *males:* β = -0.74 ± 0.23, t_{42} = -3.23, p = 0.002, Figure 2.11 b). Increases in number of flights resulted in slower flight speeds for both sexes (LME accounting for date flown: t = -6.25, n = 99, p <0.001, Figure 2.11 c), with a significant difference in intercept showing that for equivalent speeds, females engaged in more flights than males (LME accounting for date flown:: t = -2.46, n = 99, p = 0.02).Increases in average flight duration resulted in significant increases in average flight speed, but on average flights were faster in females (LM: $F_{3,95}$ = 9.66, p = 0.002, *females:* α = 2.02 ± 0.15, *males:* α = 1.67 ± 0.15, not shown).

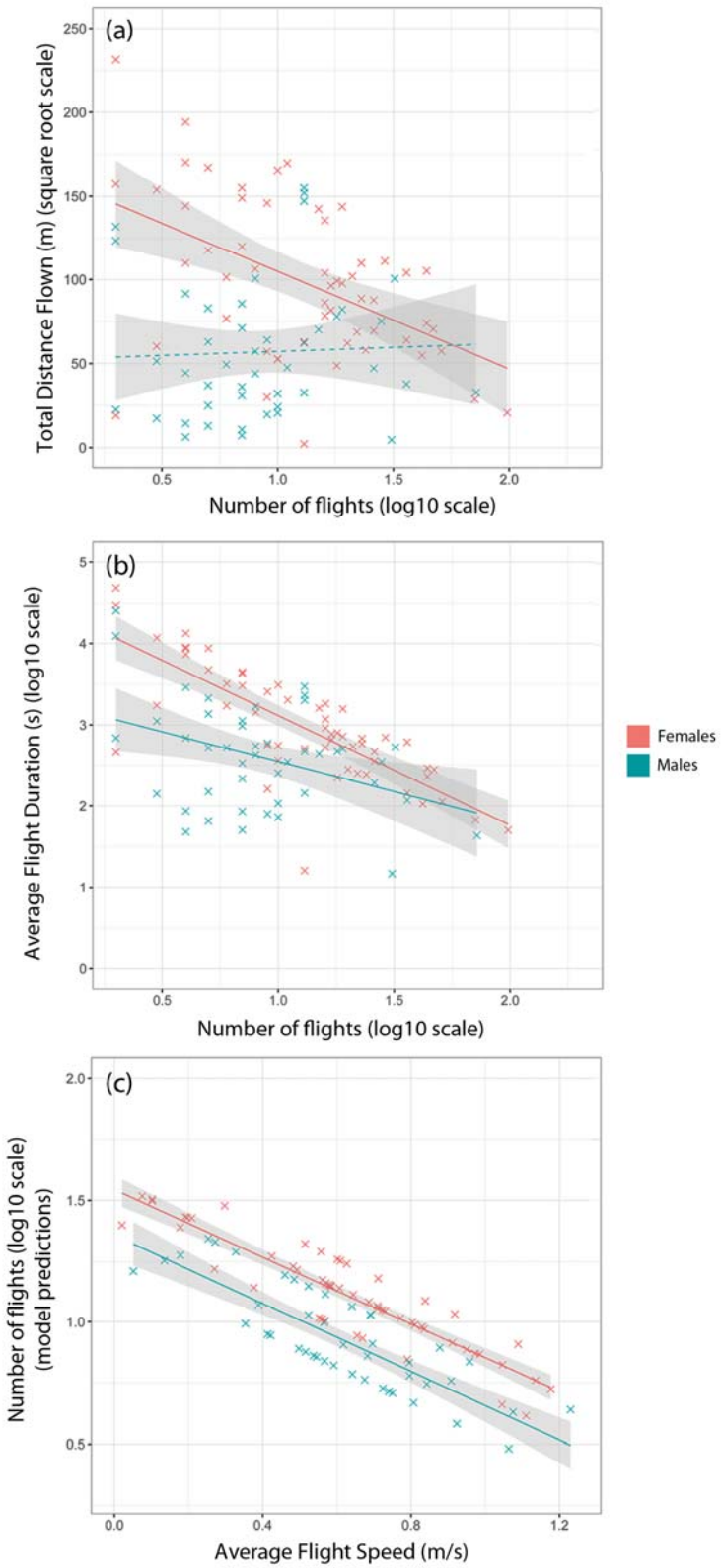

Figure 2.11: The relationship between flight variables for *S. frugiperda*. The same negative relationships found in *S. exempta* and *H. armigera* were observed in the fall armyworm. The only exception was the relationship between total distance and number of flights in males (a), which was not significantly different from zero. For the relationship between average flight duration and number of flights (b) there was an interaction between number of flights and sex, such that where females undertook the same number of flights, those flights were longer on average but the difference in duration was greatest where insects engaged in fewer flights (where insects engaged in a few hundred flights, the relationship with duration is approximately the same in both sexes) (b). The relationship between speed and number of flights also differed between the sexes, such that females which flew at the same speed engaged in more flights on average (c). Grey bands show 95% confidence intervals. Full statistics are given in the text.

For all individual flights, the positive relationship between flight duration and flight speed remained significant but was accompanied by a significant interaction between speed and sex which shows that flight duration was significantly higher in females, but flights of equivalent duration were faster in males (LME for *maximum flight speed* accounting for pseudo-replication by individual: log10 (*IndvDur*) ~ *MaxSpeed*: t = -6.4, n = 2,267, $p < 0.001$ *females*: $\beta = 1.09 \pm 0.03$, *males*: $\beta = 0.74 \pm 0.04$, Figure 2.12; LME for *average flight speed* accounting for pseudo-replication by individual: log10 (*IndvDur*) ~ *AvSpeed*: t = -6.4, n = 2,267, $p < 0.001$, *females:* $\beta = 1.43 \pm 0.07$, *males*: $\beta = 0.83 \pm 0.08$, not shown)

Full parameter estimates for all models can be found in Appendix 3.

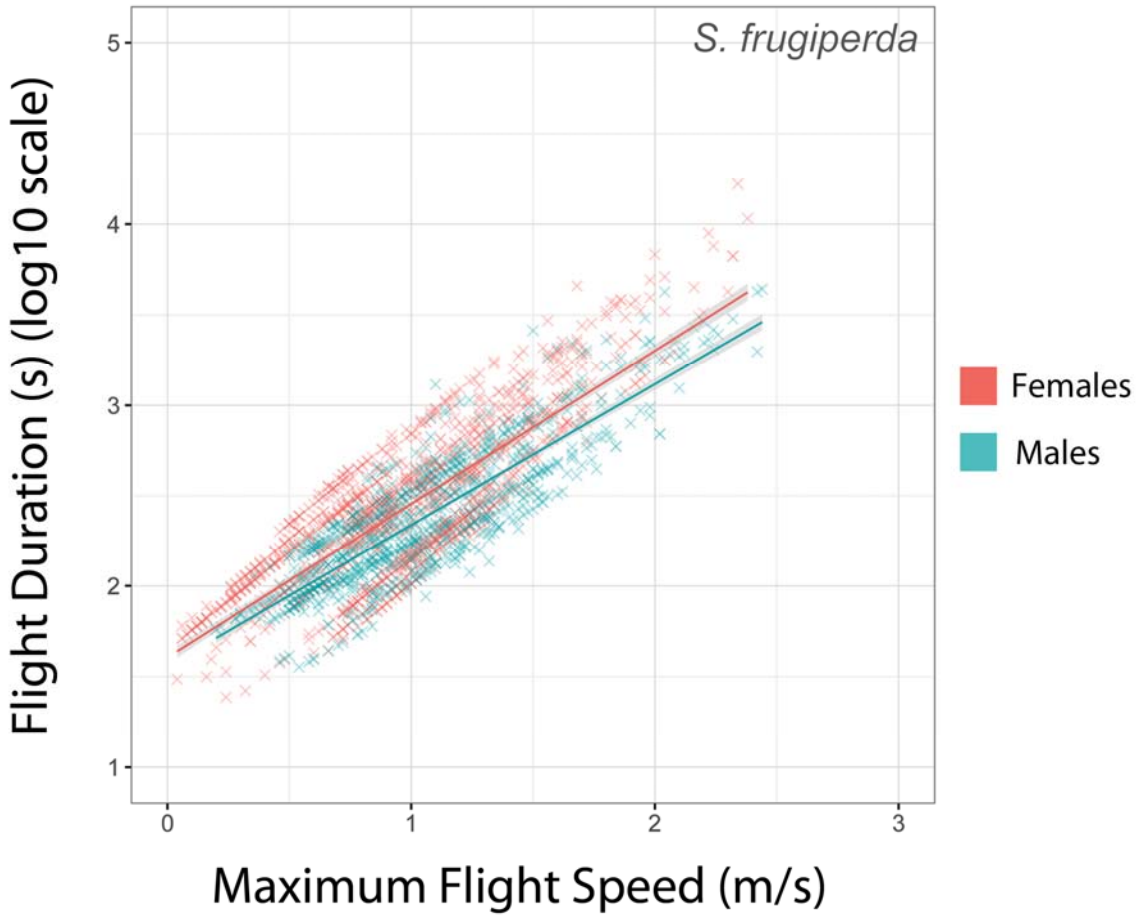

Figure 2.12: The relationship between flight speed and duration for every flight undertaken by every individual in *S. frugiperda*. The longer the flight, the faster it was on average. Speed also showed a significant interaction with sex such that females (which achieved the greatest total distances, in line with the expectation of sex-biased dispersal, (Miller et al., 2011)) had slower flight speeds for equivalent flight durations. These results are comparable to what was observed in *S. exempta* and *H. armigera* and support the definition of migration by Kennedy (1985). Significance levels can be found in the text and full model parameters are available in Appendix 3. Grey bands show 95% confidence intervals.

2.5 DISCUSSION

There are three novel findings in this chapter, each of which are dealt with sequentially below.

Firstly, in support of (Riley et al., 1997), these data provide evidence that flight mills limit the flight capacity of insects, with flight on the heavier mills resulting in reduced speeds, shorter average flight durations and, as a result, decreased total distances. In addition, the maximum speeds recorded here (circa 1 m/s) are approximately a third to a quarter of the airspeeds we expect for these species which have been recorded at 3-4 m/s using entomological radar (Chapman et al., 2011a, Drake and Reynolds, 2012). This reduction matches the results of Riley et al. (1997). What is both unexpected and novel about this, however, is that the heavier flight mills lead to an increase in the number of flights insects engaged in (Figure 2.5). This is contrary to what was hypothesised but would suggest that while the flight mills limit an insect's ability to undertake sustained flight they do not limit the underlying *drive* to fly, which is represented as an increase in the number of attempts at flight. This is relevant to the results observed in Chapter 3 (section 3.4.2).

The second finding of interest is the similarity of responses across species. It has previously been suggested by Reynolds et al. (2015) that while complex movement patterns arise in natural systems, heavily influenced by a wide range of different stimuli, there is an underlying movement template which is independent of both taxa and scale. The laboratory set up of the rotational flight mills (Figure 2.1) in a relatively featureless environment provides an excellent opportunity to test this hypothesis, and the findings here certainly support the notion that, in the relative absence of stimuli, flight in noctuid moths is underlined by a universal movement pattern. The three groupings in the principal components analysis of speed, distance/duration and number of flights (Figure 2.2, Figure 2.3 and Figure 2.4) replicate findings in Jones et al. (2016), suggesting this is common across more than 24 different noctuid moth species from across a wide range of geographic locations (the UK, China and North America) with very different dispersal strategies.

Given the migratory biology of these three species, which were all flown in the pre-reproductive period within one or two generations of being collected from the field, the similarity in movement templates across the three different migratory species considered in this Chapter are in support of the theoretical definition of migration as the persistent, straightened out movement in absence of response which s dependant on the temporary inhibition of station keeping responses (Kennedy, 1985). In all three migratory species, average flight duration, average speed and total distance were negatively correlated with the number of flights, such that individuals which flew the furthest achieved this distance by engaging in fewer, faster flights (Figure 2.7, Figure 2.9 and Figure 2.11). The relationship between speed and distance seems almost counter-intuitive and

contrasts with what was hypothesised (hypothesis ii, b and c) as we might expect individuals which are travelling greater distances to conserve their energy and travel at slower speeds. Similar increases in flight speed with increasing distance however have been observed in migratory birds and agree with optimal migration theory (Sorte et al., 2013, Nilsson et al., 2013). As the positive relationship between speed and duration was observed both across sample populations and within the flight behaviour of individual insects (Figure 2.8, Figure 2.10 and Figure 2.12), this data may even go some way to extending the definition as it would seem to provide evidence that, at least at the level of movement in a featureless environment, this migratory behaviour exists on a continuous scale rather than as a binary 'migratory' or 'non-migratory' response.

The third outcome is that the migratory response observed on the flight mills is in agreement with both the upregulation of genes associated with the migratory syndrome in *H. armigera* (Jones et al., 2015) and evidence of sex-biased dispersal in the fall armyworm (Chapter 3). The results in *S. frugiperda* should be considered within the experimental manipulations of Chapter 3, which found that the flight response to virus exposure differed between the sexes. However, this impact of the pathogen was *in addition* to a significant effect of sex (specifically, females engaged in fewer flights of longer average duration, and achieved greater distances than males while also demonstrating a differing response to infection; see Chapter 3), suggesting that while viral infection can affect migratory behaviour (Chapter 3) the variation should also be attributed to characteristics associated with sex-biased dispersal. In *H. armigera*, insects sampled from Dafeng were shown to fly significantly further on average than insects from Anyang (insects from Jingzhou and Qiuxian were statistically indistinguishable from other sample populations) (Jones et al., 2015). When compared to individuals from Anyang, insects from Dafeng also showed a significant increase in the up-regulation of genes associated with the migratory syndrome, including those which regulate the development of muscle structure, the mobilization of lipids and hormones that are known to influence the migratory phenotype. For these two groups (females in *S. frugiperda* and the Dafeng sub-population in *H. armigera*) greater distances were again achieved by undertaking fewer flights of greater duration and at increased speeds (Figure 2.9 and Figure 2.11). Within-individual comparisons also showed that flights of equivalent duration were faster in females and insects from Dafeng (Figure 2.10 and Figure 2.12). However, the relationship is not significantly different for males (*S. frugiperda*) or the Anyang population (*H. armigera*) at lower flight speeds (the 95% confidence intervals overlap). This suggests that while on average females (*S. frugiperda*) and insects from Dafeng (*H. armigera*) invest in faster, longer flights, they do so by moderating the speed only of long distance flights. Shorter flights, which may be more representative of local rather than highly dispersive movements, are the equivalent in both sexes and for all geographic populations.

In conclusion, this work builds on that of (Reynolds et al., 2015) and provides strong evidence an underlying movement template that is independent of species in migratory noctuid moths, provides strong support for the theoretical definition of insect migration (Kennedy, 1985) and is linked with factors associated with the migratory syndrome (Dingle, 2014). It also validates studies that have used rotational flight mills quantify the underlying biotic and abiotic factors that affect in migratory tendency in the Lepidoptera, which to date have been limited in applying results beyond the framework of flight capacity. Specifically, relevant to this thesis of course is those studies which relate to infection (Chapter 3) (Bradley and Altizer, 2005, Dorhout et al., 2011). Finally, it also expands on the literature that seeks to quantify the impact of the flight mills on overall flight capacity, giving he novel insight that, while flight mills may limit overall flight capacity, there is still evidence that they capture the underlying drive to fly. This is particularly relevant to the methods and results obtained in Chapter 4.

CHAPTER 3.

MIGRATORY TOLERANCE AND BATEMAN'S PRINCIPLE: THE FLIGHT RESPONSE TO INFECTION IN THE FALL ARMYWORM

3.1 ABSTRACT

Different cost functions between migratory capacity and pathogen load are likely to result in different disease dynamics at a geographical scale. While studies have highlighted the importance of temporal and geographic co-occurrence of host and pathogen, few have questioned how sexual dimorphism in disease response might affect mate availability, mate preference and disease dynamics in migratory systems. Bateman's principle suggests that males and females should have different life-history priorities to improve differential investment strategies for reproductive success. More specifically, males should seek to increase the number of successful mating opportunities while in females, selective pressure should result in longer life-spans. This principle has been shown to result in differing immune responses between the sexes in insects, but how Bateman's principle affects the tolerance of disease in migratory host-pathogen systems has not been explicitly tested. By combining rotational flight mills with pathogen bioassays, this study seeks to address that question by specifically investigating whether pathogen challenge results in different tolerance and cost functions between sexes of the fall armyworm when challenged with the baculovirus *S. frugiperda* multiple nucleopolyhedrovirus. The results demonstrate that males and females exhibit different strategies. Females appear to engage in a form of migratory escape, reducing their development time in response to increasing levels of virus challenge but showing no change in flight capacity, a strategy that appears to be costly in terms of weight lost during flight. The opposite is observed in males, which showed no change in development time but experienced significant declines in flight capacity with increasing levels of pathogen challenge, and appeared to be susceptible to some form of migratory culling. These differences in susceptibility in response to pathogen load were underscored by differences in developmental, physiological and morphological differences in the sexes, giving the first evidence of how Bateman's principle can impact susceptibility to infection in insect migrants and result in different cost functions for the two sexes in migratory animals.

3.2 INTRODUCTION

Migration is a stressful and energetically-costly behaviour (Rankin and Burchsted, 1992), but the benefits can be considerable and may offset migration associated costs in some instances (Chapman et al., 2015b, Chapman et al., 2012). Fighting off, or living with, pathogenic infections is also costly (Wilson, 2005, Schmid Hempel, 2011). In migratory animals which have been exposed to disease, this may lead to potential trade-offs between investment in migration and investment in the resistance and tolerance mechanisms that determine host susceptibility (Råberg et al., 2007).

In migratory systems, the extent to which disease affects an animal's ability to embark on and maintain long distance movement could be described by its migratory tolerance of disease. Migratory tolerance is defined as the extent to which disease affects host fitness in terms of the behavioural, physiological or genetic adaptions linked with the migratory syndrome (Dingle, 2014) and is closely related to pathogen virulence (see section 1.5.1). Where exposure to pathogens affects physiological and behavioural adaptions associated with the migratory syndrome, changes in the levels of migratory tolerance are likely to affect the disease dynamics and spatial distribution of both host and pathogen (Chapter 1).

Most work to date in migratory insect species has focused on the effect of disease on movement capacity and dispersal ability, and there are few studies which follow infection over the hosts life history and attempt to quantify the effect of infection on the overall migratory syndrome. Yet this is important as it allows us to both understand how migratory hosts allocate resources over their life cycles to tolerate infection and identify the fitness costs that drive the changes in migratory behaviour. For example, in the monarch butterfly, infection with the protozoan parasite *Ophryocystis elektroscirrha* has been linked to smaller wing spans and lower body mass (Altizer and Oberhauser, 1999, Altizer, 2001) which affect flight performance (Bradley and Altizer, 2005). Alternatively in the European corn borer, *Ostrinia nubilalis*, the cost of infection was found to vary with the age of adult moths (Dorhout et al., 2011). In newly emerged adult moths, heavily infected individuals flew significantly shorter distances when compared with controls; a cost not evident in moths that were three days old. As newly emerged adults are likely to engage in highly migratory behaviour as a part of the oogenesis flight syndrome (see section 1.2.2) this suggests that the way in which species allocate resources depends on the relationship between reproductive status, flight behaviour and infection levels.

There are also several examples in the insect literature of different costs of infection in males and females. Variation in flight capacity for both 1 day and 3 day old moths was observed in the European Corn Borer by Dorhout et al. (2011) as well as in the monarch butterfly (Bradley and

Altizer, 2005, Davis et al., 2012) and African armyworm (Gatehouse and Hackett, 1980). Although there is little work to date studying this phenomenon in migratory insects, this would suggest that sex biased dispersal (where one of the sexes has a higher dispersal tendency than the other, (Miller et al., 2011)) may exist in some migratory lepidopteran populations where variation in susceptibility to infection between the sexes has also been observed. For example, in the monarch *O. elektroscirrha* infection leads to lower haemocyte counts, decreased lifespans and reduced reproductive success in males but not females (Altizer and Oberhauser, 1999, Lindsey and Altizer, 2009), while in the European corn borer infection has been associated with differential effects in flight capacity between the two sexes (Dorhout et al., 2011).

In disease ecology, differences in the response to infection between the sexes is a well-known phenomenon, which is partially attributed to Bateman's principle (Bateman, 1948). This principle assumes that the factors which affect the reproductive success of males and females differ: males stand to gain the greatest advantage from investing in mating success while females should invest in reproductive effort, and this puts different selective pressures on their life-history traits (Schmid Hempel, 2011). This has led to predictions that females should invest more in immunity than males (Rolff, 2002, Moore and Wilson, 2002). In insects there are meta-analyses that would suggest that females do invest more in certain immune functions such as phenoloxidase activity than males (Nunn et al., 2009).

While differences in migratory capacity between the two sexes have been observed in some cases, the extent to which this relates to different cost functions and, more specifically, differential investment in life history strategies and disease tolerance in migrants has not been specifically tested. This chapter addresses that question by using rotational insect flight mills (Chapter 2) and pathogen bioassays to quantify how the flight capacity and life history strategies of the fall armyworm *Spodoptera frugiperda* vary between sexes when infected with the baculovirus *S. frugiperda* multiple nucleopolyhedrovirus.

To that end, this chapter has two specific aims. Firstly, it uses the approach described by (Chapman et al., 2015b) (see section 1.5.8, Figure 3.1) to quantify the disease cost function across multiple behavioural and physiological characteristics associated with the migratory phenotype (weight, development time, wing length and flight capacity). As the response to infection can vary over time, with the host and pathogen moving through a disease cycle that can include stages such as parasite growth, pathogenesis and recovery, each of which may carry varying costs (Schneider, 2011), and this is particularly relevant when investigating the sublethal effects of baculoviruses in adult noctuid moths, as infection occurs in the larval stage and studies have shown that disease levels change throughout the insects' life cycle (Graham et al., 2015). To

quantify the impact of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (*Sf*MNPV) on migratory tolerance of the fall armyworm, I tested the following hypotheses:

i. Increases in pathogen challenge will lead to decreases in development time (Cabodevilla et al., 2011b);
ii. Adult body weight will decrease with increasing level of exposure to baculovirus infection (Cabodevilla et al., 2011b); and
iii. The cost of infection on development and weight will also impact morphological characteristics (specifically wing length)

The second aim of this chapter was to assess if variation in disease tolerance (the impact of infection on weight, development time, wing length and flight capacity) within a species can be explained by Bateman's principle. The specific hypotheses tested were:

iv. Females will invest more in immunity than males and prioritise migratory escape, while males will be more susceptible to infection and exhibit a reduction of flight capacity with increasing levels of *Sf*MNPV virus; (Dorhout et al., 2011)
v. Increases in pathogen challenge will lead to decreases in development time in females ('migratory escape') but not males (Dorhout et al., 2011)
vi. Adult body weight will decrease with increasing level of exposure to baculovirus infection in females but not males, accounting for differences in immune response (Altizer and Oberhauser, 1999); and
vii. The cost of infection on development and weight will also impact morphological characteristics (specifically wing length) (Bradley and Altizer, 2005), and this will vary in the two sexes.

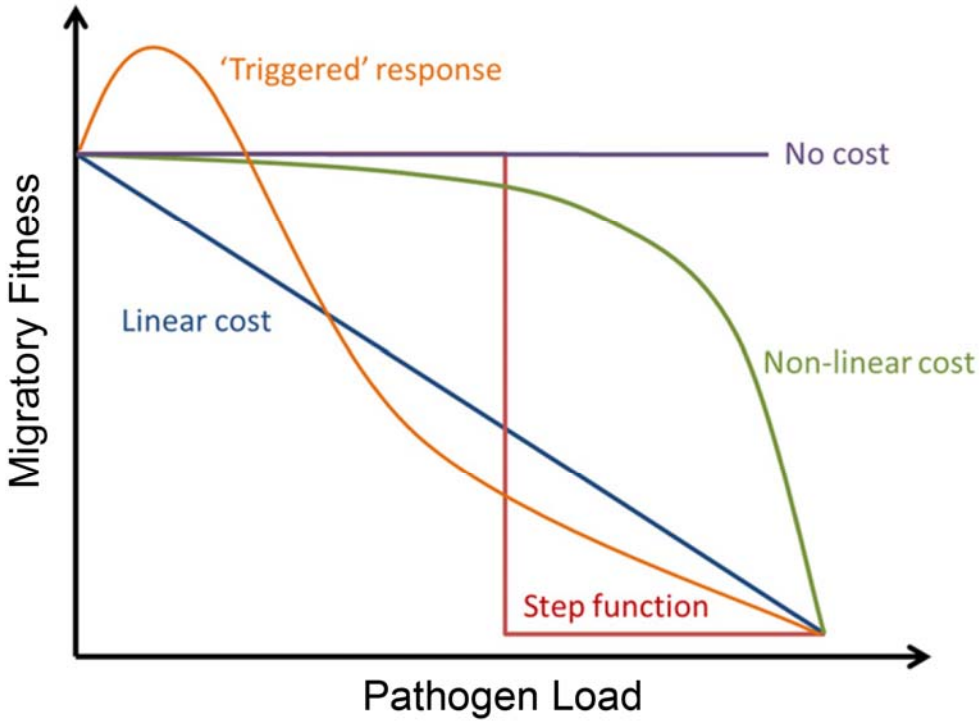

Figure 3.1: Theoretical cost functions for migratory tolerance Migratory tolerance can be defined as the effect of pathogen infection on fitness parameters associated with the migratory syndrome. As pathogen loads increase, we might expect there to be a cost in terms of migratory fitness. This cost may be linear (blue line), nonlinear (green), a step function (red) or negligible (purple); low-level infections may also act as a cue that triggers a short term reallocation of resources which result in an increase in fitness, enabling migratory escape (orange). This chapter explores the cost functions of *Sf*MNPV infection in the fall armyworm on multiple behavioural and physiological characteristics (flight capacity, weight and development time) associated with the migratory phenotype, and compares these across the sexes to determine if Bateman's principle affects migratory tolerance in this host-pathogen system. Text and figure adapted from Chapman et al. (2015b).

3.3 METHODS

3.3.1 INSECT CULTURES

Larvae were collected from cornfields in Belle Glade, Florida in mid-January 2014 by the United States Department of Agriculture (USDA ARS) in Gainesville, Florida. Insects were shipped to Rothamsted Research, UK as pupae and reared on semi-artificial diet under controlled conditions at 26 °C ± 2 °C, with a 16 h light: 8 h dark lighting regime (see appendix 2 for full rearing protocol). The culture was reared through a clean generation and, as no overt signs of baculovirus infection were evident, the third laboratory generation were used in this trial.

3.3.2 PATHOGEN CHALLENGE

Insects were infected with a purified wild-type strain of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (*Sf*MNPV) isolated from Nicaragua (*Sf*NIC) and provided by the Universidad Pública de Navarra in Pamplona, Spain (Escribano et al., 1999, Simon et al., 2004a). Viral occlusion bodies (OBs), which can be thought of as individual infectious units (Chapter 1) were counted using a Neubauer haemocytometer (P/N DHC-NO1, NanoEnTek, Korea). The resulting concentration was diluted in autoclaved, distilled water to create a 10-fold series dilution of 10, 100, 1,000, 10,000 and 100,000 OBs/µl. These formed the five virus treatment groups and a sixth treatment of autoclaved distilled water was used as a control.

Egg batches were collected over a period of four days from mating cages that contained approximately 40 non-sibling mating pairs. Eggs were left to hatch in 350ml containers lined with artificial diet and monitored daily. On the day that they were observed to be fourth instar, larvae were randomly allocated to a treatment group. Insects were orally challenged by placing each caterpillar in the well of a 96 well NUNC-Immuno™ MicroWell™ plate (P/N 260836; Thermo Scientific™, UK) and providing them with a diet plug that contained 1µl of the OB suspension (Graham et al., 2012). Insects were left to consume the diet overnight. Any larvae that did not consume the diet plug were removed from the trial. To reduce the potential for contamination, treatments were kept separate and the control was handled first, working up through the series dilutions. Each well was only used once.

After inoculation, insects were reared in individual 37ml pots (Pot P/N: T125-990; Lid P/N: Pl1N, Solo, UK) lined with semi-artificial diet. Two batches of artificial diet were prepared for this trial. As nutrient uptake can play a key part in resistance to infection and to control for any differences in preparation, the two diet batches were divided equally between treatments and randomly assigned among individuals within treatment groups. Diet batch was also accounted for as a random term in the mixed effects models where appropriate.

3.3.3 FLIGHT

Insects were monitored daily and the date of pupation, pupal weight five days after pupation, the date of adult emergence and any deaths and their causes were recorded. Moths were flown on rotational flight mills within 24 h of emerging from their pupal case using the procedure described in Chapter 2. Two hours before being attached to the flight mills, insects were weighed, fed *ad libitum* with a 10% sucrose solution and weighed again to calculate any weight gain. Insects were placed on the flight mills between 18:30 and 19:30 GMT and removed between 9:00 and 10:30 GMT the following morning. After being removed from the flight mills the moths were weighed again so weight loss could be calculated. Insects were euthanised by placing them in the freezer the morning after flight and then stored in ethanol at -20˚C.

3.3.4 WING LENGTH

Wings were separated from the thorax using a scalpel and placed between two microscope slides to keep them flat while taking measurements. The length of both wings (Figure 3.2) was measured using digital callipers, accurate to the nearest 0.01 mm. Wing length was modelled as the average of both wings except where one of the wings was damaged, when the length of only one wing was used.

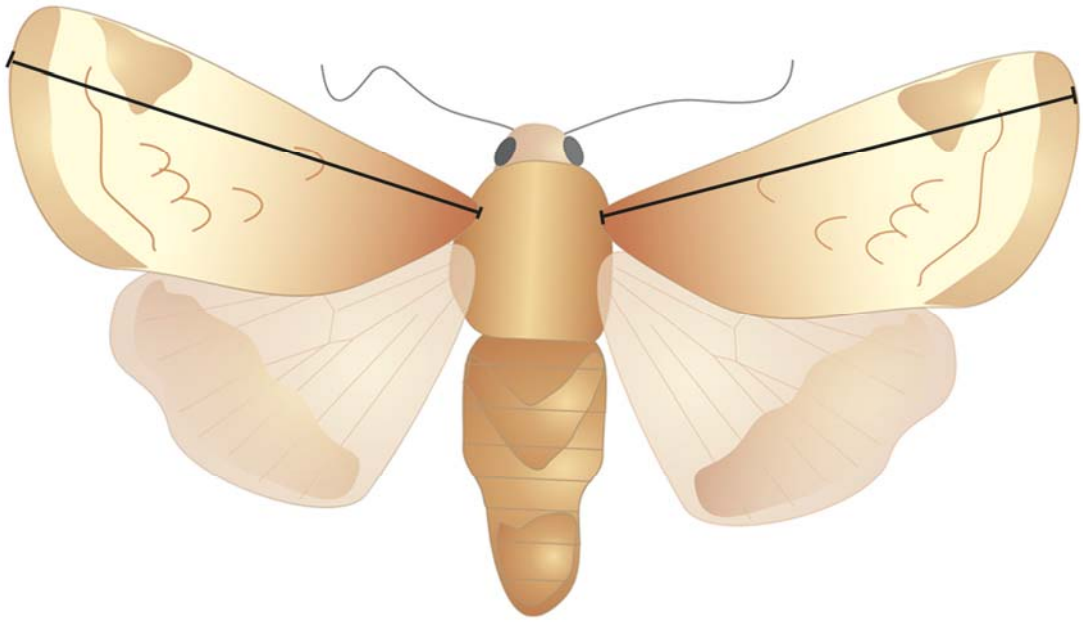

Figure 3.2: Diagram of wing measurements. The longest section of both wings (black lines) were measured using digital callipers accurate to 0.01 mm and the average used in analysis. Damaged wings were not measured, and in these instances the analysis only used measurements from one wing.

3.3.5 STATISTICAL ANALYSIS

Pathogen challenge is likely to affect multiple aspects of an individual's life history, physiology and behaviour. Using the statistical modelling process described in Chapter 2, linear mixed effects models (LMEs; assuming a normal distribution of data) were used to investigate the impact of pathogen challenge on the development (section 3.3.5.3), adult weight (section 3.3.5.4), flight behaviour (section 3.3.5.2) and wing length (section 3.3.5.5) of the fall armyworm.

3.3.5.1 MORTALITY

As the aim of this study was to assess sub-lethal effects of viral infection, the trial was designed to ensure high levels of survival in the host. More than one cause of death was evident in the culture (in addition to overt baculovirus infection, there were insects which failed to pupate or died during pupation). To assess how all causes of death related to virus challenge, each was individually investigated using a general linear model (GLM) with a binomial error structure:

> Logit (*proportional mortality due to a specific cause of death*) = α + β x *pathogen challenge* (log10 - transformed)

3.3.5.2 FLIGHT BEHAVIOUR

To understand the effect of pathogen challenge on overall variation in flight behaviour, the scores for the first two principal components (PC1 and PC2) were extracted from principal components analysis (PCA) of *S. frugiperda* in Chapter 2. The score for an individual insect comes from multiplying the loading for each flight variable by the result attained by that individual. To give a single measurement of overall performance, the outcome for all sixteen flight variables included in the PCA is then added together to form a linear transformation of an individual's general performance. As such, these scores should be interpreted as an overall estimate of the variability in the data, each point representing an individual's contribution to the variation described in the principal component.

However, PCA does not give specific insights into the actual behaviour or flight capacity of an individual. To investigate this, flight capacity (total distance flown) and flight behaviour (average flight duration, maximum flight speed and number of flights), were modelled as a function of the interaction between pathogen challenge and sex, considering adult weight prior to flight, weight gained during feeding and total development time (TDT). The starting model for all variables was:

> *Flight variable* (transformed as appropriate) = α + β1 x *pathogen challenge* (log10-trasnformed) + β2 x *sex* + β3 x *larval weight post infection* (mg) +
> β4 x *adult weight prior to flight* (mg) + β5 x *weight gained from feeding* (mg)+
> β6 x *pathogen challenge* (log10-transformed) x *sex*

Transformations differed for individual flight variables and are listed in Chapter 2 (Table 2.1). To account for variation introduced as part of the experimental design random terms investigated were: day flown (equivalent to the date of adult emergence), sibling group (as above), diet batch and mill set (two banks, each containing a set of 16 flight mills. See Chapter 2).

3.3.5.3 DEVELOPMENT

The effect of pathogen challenge on development time was investigated for larvae (larval development time or LDT; defined as the number of days between infection and pupation) and pupae (pupal development time or PDT; defined as the number of days between pupation and adult emergence) as well as for the TDT which was defined as the number of days between infection and adult emergence.

All three responses were modelled using linear mixed effects models (LME, see Chapter 2) and investigated the interaction between sex and dose, taking into account the weight of larvae immediately post-infection. The starting model for TDT and PDT was:

> *Development time (days)* = α + β1 x *virus dose* (log10-transformed) + β2 x *Sex* + β3 x *larval weight post infection (mg)* + β4 x *pupa weight* (mg) + β5 x *virus dose* (log10-transformed) x Sex

For LDT, pupal weight was removed from the starting model. Random terms included the day insects were infected, sibling group (refers to the egg batch collected and reared gregariously until infection) and diet batch.

3.3.5.4 WEIGHT

In this study, four different weight variables were tested using linear mixed effects models (Chapter 2): pupal weight; adult weight at emergence; proportional weight gained during feeding; and proportional weight lost after feeding and flight. The starting model investigated the interaction between sex and pathogen challenge, taking into account larval weight at infection:

> *Weight variable* (mg) = α + β1 x *pathogen challenge* (log10-transformed) + β2 x *Sex* + β3 x *larval weight post infection* (mg) + β4 x *pathogen challenge* (log10-transformed) x *Sex*

To account for structural variation, random terms tested in the model were sibling group and diet batch. Infection date was used as a random term for pupal weight and pupal weight loss, but for all other flight variables this was replaced with date flown.

3.3.5.5 WING LENGTH

To assess the relationship between wing length and flight behaviour, the four flight variables (total distance flown, average flight duration, maximum flight speed and number of flights) were

modelled as a function of the interactions between (1) wing length and pathogen challenge and (2) wing length and sex. This gave the starting model:

> *Flight variable* (transformed as appropriate) = α + β1 x *wing length* (mm) + β2 x *pathogen challenge* (log10-transformed) + β3 x sex + β4 x *wing length* (mm) x *pathogen challenge* (log10-transformed) + β5 x *wing length* (mm) x sex

Random terms investigated to account for variation introduced by the experimental design were day flown, sibling group, diet batch and the two banks of mills used. As the interaction between sex and pathogen challenge on flight behaviour was investigated above, the individual sex and pathogen challenge terms were removed from the model if the interaction was not significant.

Wing length was also modelled as a function of total development time (TDT), weight and sex (referred to as developmental and physiological variables or DP variables) and the interaction between DP variables and sex*:*

> *Wing length* (mm) = α + β x *DP variable* + β x *pathogen challenge* (log10-transformed) + β x *sex* + β x *DP variable* x *pathogen challenge* (log10-transformed) + β x *DP variable* x *sex*

Random terms included were sibling group and diet batch.

3.4 RESULTS

3.4.1 MORTALITY

As the aim of this study was to investigate sub-lethal effects of a pathogen challenge on flight, the virus doses used were designed to ensure that the majority of insects survived to adulthood. Of the 327 larvae infected, 220 (67.3%) survived to adult emergence. The survival rate for NPV infection was very high with only 14 larvae (4.3%) in the two highest virus doses dying of overt baculovirus infection. There were no NPV-related deaths in the control group, and as expected pathogen challenge correlated with NPV-related mortality (Binomial GLM: $Z = -3.44$, $p < 0.001$, Figure 3.3). Most those deaths were the result of larvae failing to pupate (23.2%) or dying during pupation (5.2%). For both causes of death prior to or during pupation, there was no significant relationship with pathogen challenge ($p > 0.05$).

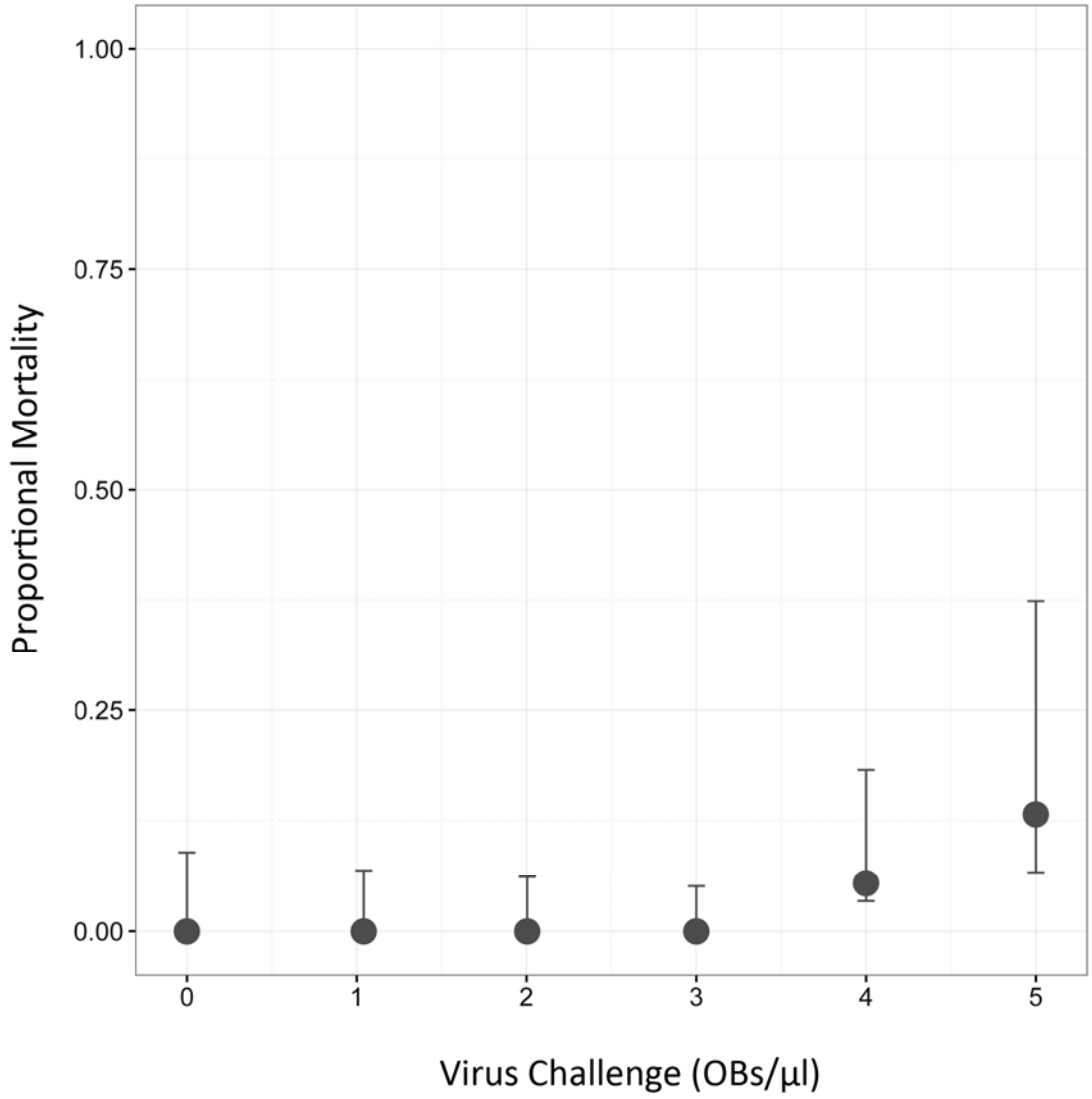

Figure 3.3: NPV-related mortality as a result of pathogen challenge. The proportion of insects succumbing to *Sf*MNPV infection (virus isolate: *Sf*NIC). Error bars show 95% binomial confidence intervals, calculated using the Clopper-Pearson exact confidence interval formula (Julious, 2005).

3.4.2 FLIGHT BEHAVIOUR

Susceptibility to disease in migratory systems could be considered to occur in two different forms: the impact on overall flight capacity (defined as the overall ability of an animal to fly) and the effect on flight behaviour (a suite of different movement traits under an individual's control, for example speed, direction, duration and number of flights) (see Chapter 2). To investigate the effect of viral challenge on both measures of flight susceptibility, and how this relationship might vary as a result of sex (hypothesis 1), two different statistical techniques were used.

The first analysis which investigated the effect of pathogen challenge on flight capacity used principal components analysis (PCA). This technique is a means of combining and transforming all possible measures of flight behaviour into individual variates (principal components), each of which summarises a proportion of the total variation that occurs across all behaviours. For each principal component, every individual is given a score that describes its contribution to the variation observed (Table 3.1). In this study, the first two principal components (PC1 and PC2) explained 72.77% and 9.75% of the total variation in flight behaviour. To understand how this variation changed in response to the interaction between sex and virus challenge, the scores were then modelled as described using linear mixed effects models (LMEs). The results showed that there was a significant interaction between viral challenge and sex for PC1 (LM: $F_{3, 95} = 4.82$, $p = 0.030$, Figure 3.4), which shows no effect of viral challenge on the flight behaviour of females ($t_{53} = 0.243$, $p = 0.809$, Figure 3.4a) but significant changes in the flight behaviour of males with increasing viral dose ($t_{42} = 2.70$, $p = 0.010$, Figure 3.4b). Sex, but not viral dose, also explained significant variation in flight traits associated with PC2 (LM: $F_{1,97} = 6.18$, $p = 0.015$). Thus, there were clear sex differences in flight capacity, as also shown in chapter 2, but there were also sex differences in how flight capacity was affected by sub-lethal viral infection.

The second analysis looked specifically at the effect of pathogen challenge and sex on flight behaviours. Based on observations in Chapter 2, four different flight variables were modelled as a function of the interaction between virus challenge and sex: total distance flown, average flight duration, maximum flight speed and number of flights. The effect of virus challenge on flight behaviour varied depending on the flight variable in question. For average flight duration (LM: $F_{3,95} = 6.74$, $p = 0.01$), total distance flown (LM: $F_{3,112} = 5.38$, $p = 0.022$, Figure 3.4c,d) and number of flights (LME with sibling group and date flown as random terms: $F = 9.87$, $n = 99$, $p = 0.002$) there was a significant interaction between viral dose and sex. For each of the interactions, there was no significant effect of viral challenge on flight behaviour in females ($p>0.05$), while in males the nature of the interaction depended on the flight variable (Figure 3.5). Males showed a significant negative linear decline in average flight duration ($t_{42} = -2.74$, $p = 0.009$, Figure 3.5b) and total flight distance ($t_{58} = -2.16$, $p = 0.035$, Figure 3.5b), and a significant polynomial response

for the number of flights undertaken ($t_{42} = 3.15$, $p = 0.003$, Figure 3.5d). In combination with average flight duration, this showed that males in the control and lowest level infection groups undertook few flights of long duration. At intermediate doses, the number of flights increased while the duration decreased slightly and at the highest levels of pathogen infection, males undertook fewer flights that were of a shorter distance. This contributed to the decrease in total distance flown with increasing pathogen challenge observed in males. In both sexes, there was no effect of any of the predictor variables on maximum flight speed ($p > 0.05$, Figure 3.5e, f). As such, pathogen challenge was shown to significantly affect overall flight capacity by modulating the behaviour that individuals engaged in.

Flight Variable	**Transformation**	**PC1**	**PC2**
Total Distance Flown (s)	square-root	-0.25	-0.16
Average Flight Distance (s)	log10	-0.27	-0.09
Total Flight Duration (s)	square-root	-0.24	-0.23
Average Flight Duration (s)	log10	-0.27	-0.18
Number of Flights	log10	0.11	0.07
Maximum Flight Speed (m/s)	-	-0.22	0.45
Average Flight Speed (m/s)	-	-0.18	0.16
Proportion of flights above 1000s	square-root	-0.22	-0.21
Maximum Speed of Longest Flight (m/s)	-	-0.23	0.39
Distance of the Longest Flight (m)	log10	-0.28	-0.05
Duration of the Longest Flight (s)	log10	-0.27	-0.16
Maximum Speed of the Furthest Flight (m/s)	-	-0.23	0.39
Distance of the Furthest Flight (m)	log10	-0.28	-0.06
Duration of the Furthest Flight (s)	log10	-0.27	-0.15
Maximum Speed of the Fastest Flight (m/s)	-	-0.22	0.45
Distance of the Fastest Flight (m)	log10	-0.27	-0.08
Duration of the Fastest Flight (s)	log10	-0.26	-0.16
Importance of components:			
Standard deviation of individual scores:		3.52	1.29
% Variance Explained:		72.8%	9.7%
Cumulative Variation Explained:		82.5%	

Table 3.1: Contributions of individual flight variables to Principal Components. The 17 flight variables used in the principal components analysis (PCA) and their respective transformations. The two columns on the right give the contribution of each of the flight variables to the two principal components investigated in this study (PC1 and PC2, see text). This shows that the contribution of each of the flight variables to PC1 was broadly similar, while in PC2 the greatest contributor to the total variation were speed variables. Also given is the standard deviation of the scores for all individual insect replicates, the percentage of the total variation in flight capacity explained by PC1 and PC2, and the cumulative variation explained by the two principal components together.

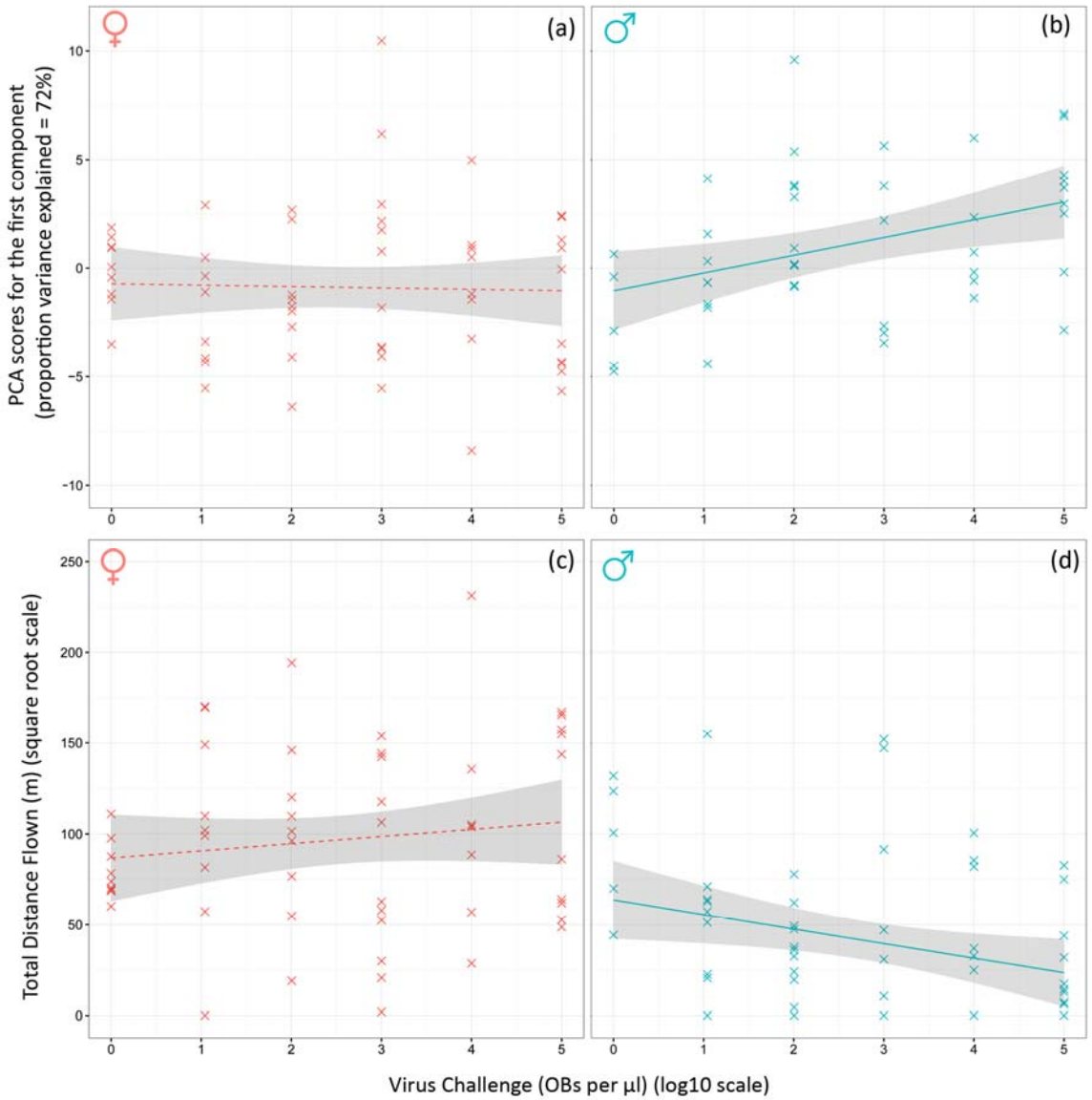

Figure 3.4: The effect of pathogen challenge on flight capacity and overall variation in flight behaviour. Overall variation in flight behaviour was described by a significant interaction between pathogen challenge and sex, with females (a) showing no change in overall variability, while the nature of the variability increased in males with increasing levels of exposure (b). This translated into significant differences in flight capacity (total distance flown, bottom), which was unaffected in females (c) but showed a decline at higher levels of pathogen challenge in males (d). Statistics are given in full in section 3.4.2. Grey bands represent 95% confidence intervals.

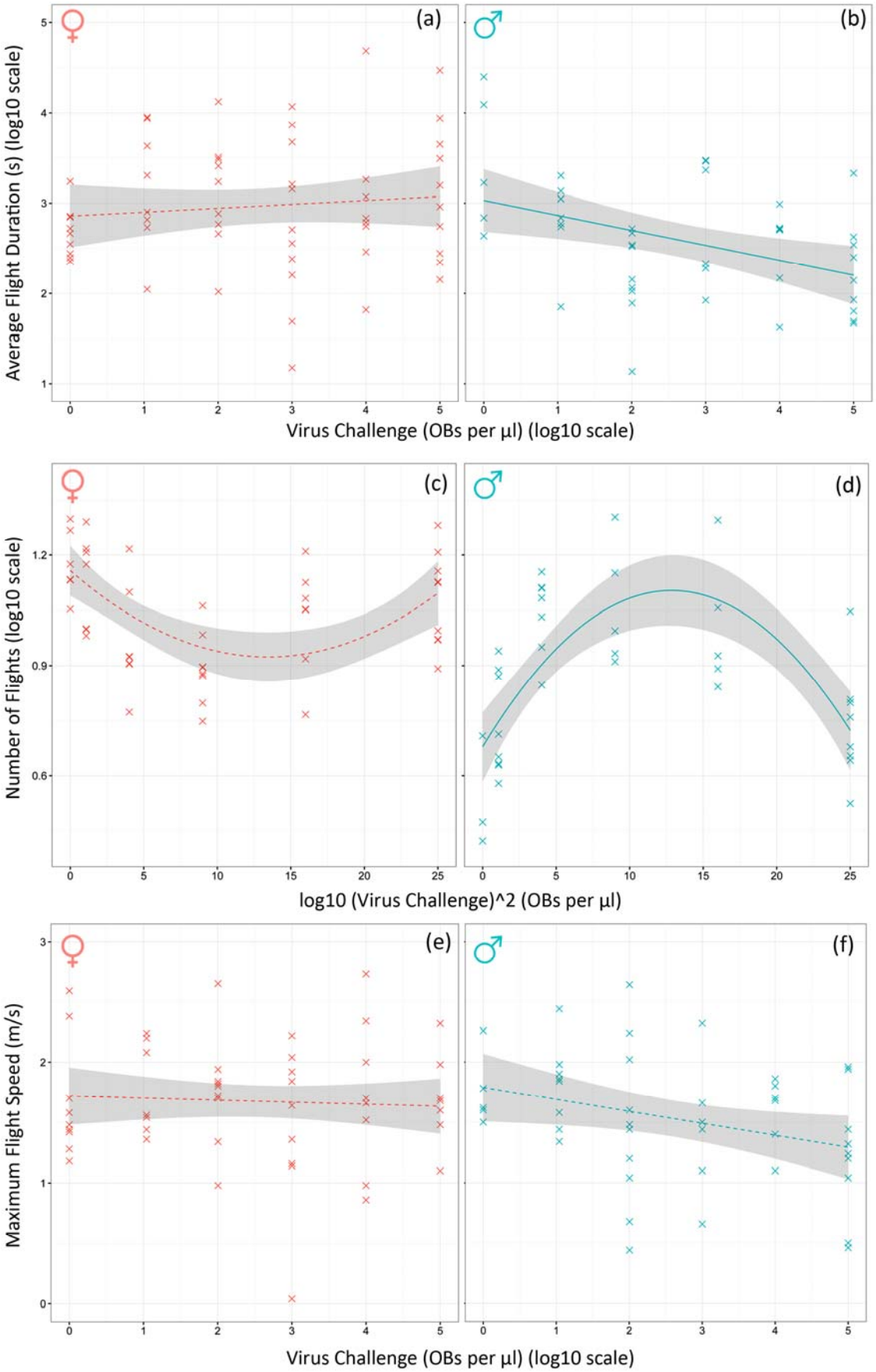

Figure 3.5: The effect of pathogen challenge on flight behaviour. Virus challenge had no significant effect on the flight behaviour of females (a, c, e) and in males the effect differed depending on the flight variable. Both flight speed and average flight duration showed a linear cost, while the cost function for number of flights is a nonlinear power law (figure 3.1). In combination these results show that in the controls and at low levels of pathogen challenge, males undertook few flights with a higher average duration (b,d). At intermediary doses, average duration decreased further but the insects increased their number of flights, suggesting an underlying drive to fly which could not be maintained. At the highest levels of infection, moths undertook very few flights with the shortest average duration. Statistics are given in section 3.4.2. Grey bands show 95% confidence intervals.

3.4.3 DEVELOPMENTAL TIME

The second hypothesis in this chapter assumed that susceptibility to virus challenge would affect other aspects of the insect's life history strategy, including a reduction in development time (Cabodevilla et al., 2011b). Holometabolous insects experience extreme changes in their physiology during metamorphosis, and may have different responses to infection at different life history stages (Russell and Dunn, 1996, Thomas and Rudolf, 2010). As such the life history stage of the insect may vary in its response to infection, and this was assessed using linear mixed effects models (LMEs) to investigate the interaction between sex and pathogen challenge in larvae, pupae and across the entire life span of the insects from infection to adult emergence.

The effect of virus dose on development time differed depending on the life history stage of the insect. In larvae, the only significant predictor of development time was larval weight at infection (LME with diet batch as a random term: $F = 68.96$, $n = 210$, $p < 0.001$), with heavier larvae developing fastest (Figure 3.6). In pupae however, there was a significant effect of pathogen challenge, where insects that were exposed to an increasing viral dose as larvae developed faster as pupae (LME with sibling group as a random term: $F = 11.98$, $n = 210$, $p < 0.001$, Figure 3.6). There was also a significant difference between the sexes, with females developing faster than males in the pupal phase (LME with sibling group as a random term: $F = 283.12$, $n = 210$, $p < 0.001$, Figure 3.6). The interaction between these two factors was non-significant ($p > 0.05$).

When the two development periods were combined to quantify total development time post-challenge (TDT), sex (LME with sibling group and diet batch as random terms: $F = 6.39$, $n = 210$, $p = 0.012$) and virus dose (LME with sibling group and diet batch as random terms: $F = 4.08$, $n = 210$, $p = 0.045$) remained significant terms in the model and there was an additional significant interaction between sex and virus dose (LME with sibling group and diet batch as random terms: $F = 4.11$, $n = 210$, $p = 0.044$, Figure 3.7), with females developing faster in response to increasing pathogen challenge ($t_{95} = 2.79$, $p = 0.006$). This additional effect of pathogen challenge was not evident in males ($t_{111} = 0.02$, $p = 0.984$). Finally, insects that took longer to develop post-challenge also weighed more as pupae (LME with sibling group and diet batch as random terms: 19.95, $n = 10$, $p < 0.001$, Figure 3.7).

In summary, the interaction between sex and pathogen challenge was shown to vary depending on the life-stage of the insect, giving an insight into the differences observed in flight capacity and behaviour.

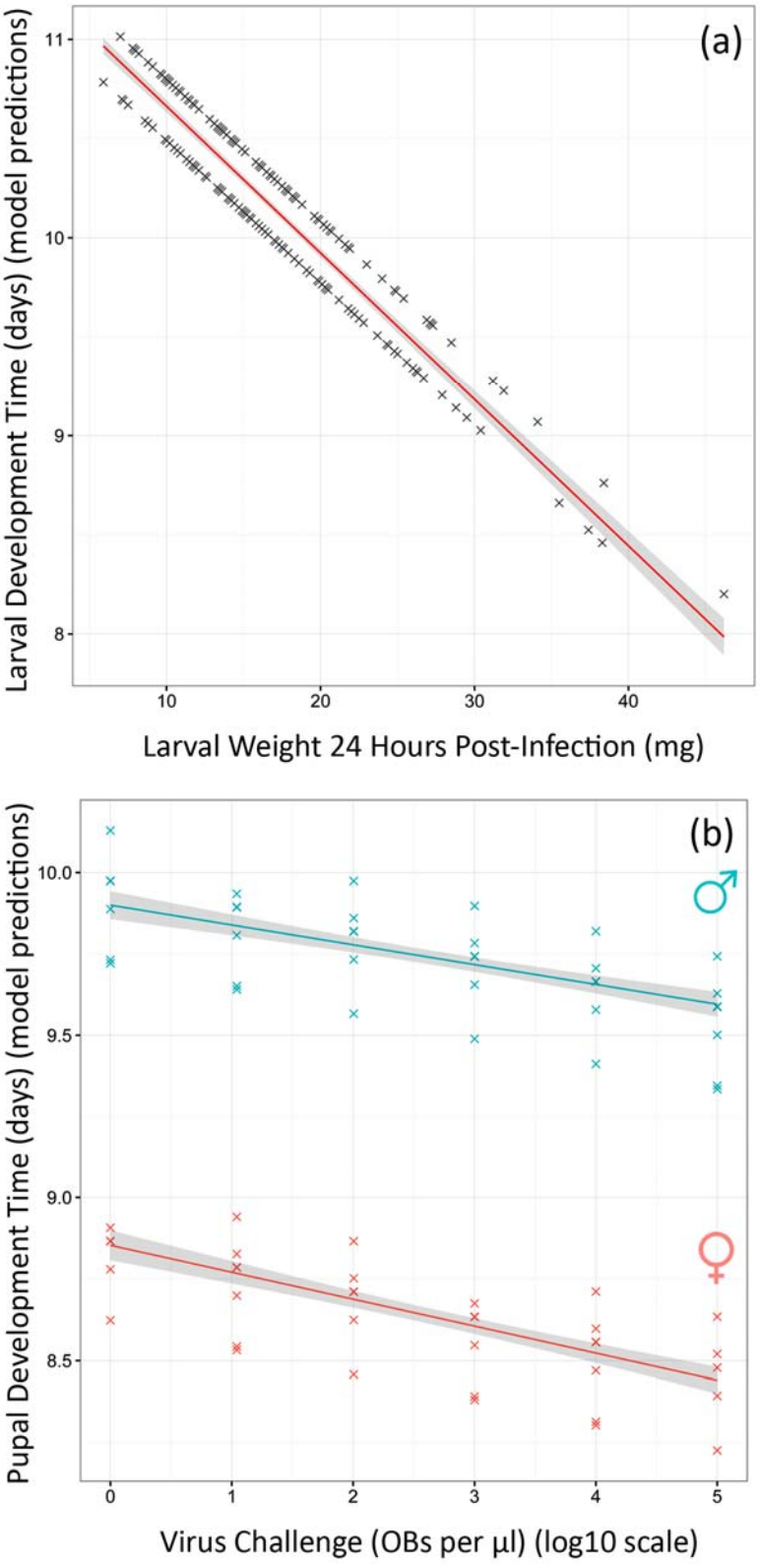

Figure 3.6: The effect of pathogen challenge on development time at different life history stages. The only significant predictor of larval development time (a) was weight at infection. In pupae (b), higher levels of pathogen challenge lead to a decrease in development time for all individuals, although females developed faster than males on average. Significance levels are described in section 3.4.2. Grey bands show 95% confidence intervals.

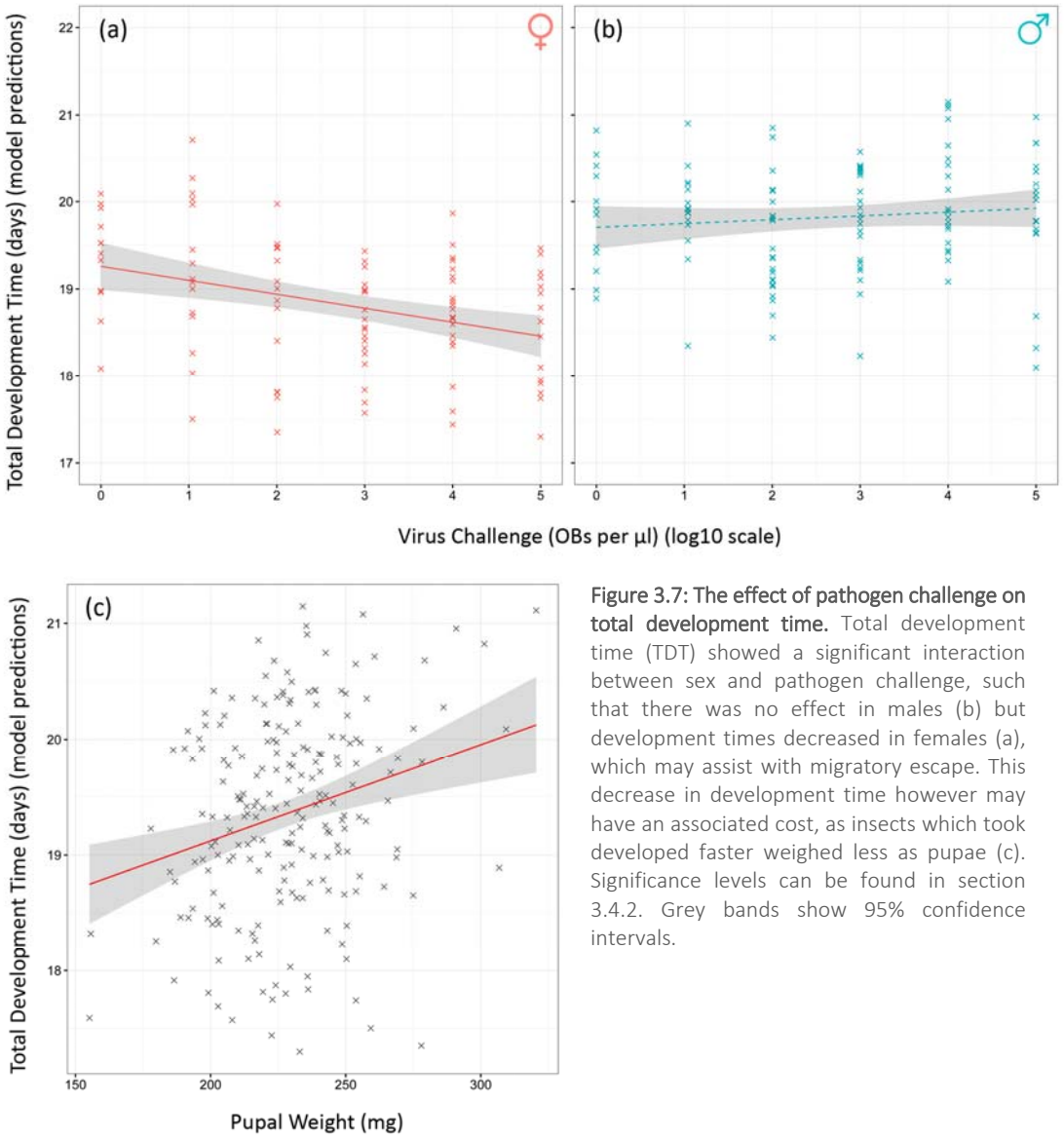

Figure 3.7: The effect of pathogen challenge on total development time. Total development time (TDT) showed a significant interaction between sex and pathogen challenge, such that there was no effect in males (b) but development times decreased in females (a), which may assist with migratory escape. This decrease in development time however may have an associated cost, as insects which took developed faster weighed less as pupae (c). Significance levels can be found in section 3.4.2. Grey bands show 95% confidence intervals.

3.4.4 Pupal and Adult Moth Weight

The weight of migratory individuals is known to have important implications for available energy reserves (Dingle, 2014a) and response to infection (Rothman and Myers, 1996). As such, weight was another variable that was expected to be affected by pathogen challenge, and was hypothesised to decrease with increases in pathogen challenge. Again, it was expected that changes in weight because the interaction between sex and virus challenge may vary with different life history stages. Consequently, insects were weighed as pupae, newly emerged adults, and pre- and post-flight. These variables were investigated with linear models (LMs) and linear mixed effects models (LMEs).

There was some evidence of differences in weight between the sexes, as well as differences in the amount of weight lost during flight as a result of the interaction between virus challenge and sex. Specifically, there was no significant effect of sex or pathogen challenge on pupal weight ($p > 0.05$), but males weighed less than females on emergence (Linear Model, LM: $F_{2,157} = 15.43$, $p < 0.001$). Females also fed on proportionally more sucrose solution pre-flight than males (LME with date flown as a random term: $F = 4.6$, $n = 126$, $p = 0.034$) and this may have influenced proportional weight loss during flight, which was described by a significant interaction between sex and pathogen challenge (LME with date flown as a random term: $F = 8.55$, $n = 135$, $p = 0.004$; Figure 3.8). This relationship was such that females lost more weight at increasing doses ($t_{60} = 2.62$, $p = 0.011$; Figure 3.8a) while there was no significant effect of pathogen challenge on weight loss in males ($t_{71} = -1.5$, $p = 0.138$; Figure 3.8b). On average, however, females still weighed more than males after flight (LM: $F_{2,136} = 6.39$, $p = 0.013$).

Larval weight at infection was a significant predictor of pupal weight (LME with sibling group and diet batch as random terms: $F = 7.53$ $n = 210$, $p = 0.007$), adult emergence weight (LM: $F_{2,157} = 5.17$, $p = 0.024$) and post-flight weight (LM: $F_{2,136} = 6.39$, $p = 0.013$). In all instances, heavier larvae weighed more as both pupae and adults.

In summary, virus challenge does not seem to affect weight at the pupal stage but does not carry the cost of increased weight loss during flight at higher levels of pathogen challenge in females (this was not significant for males). A portion of this weight loss in females might be attributable to the amount of weight gained from feeding on sucrose solution prior to flight.

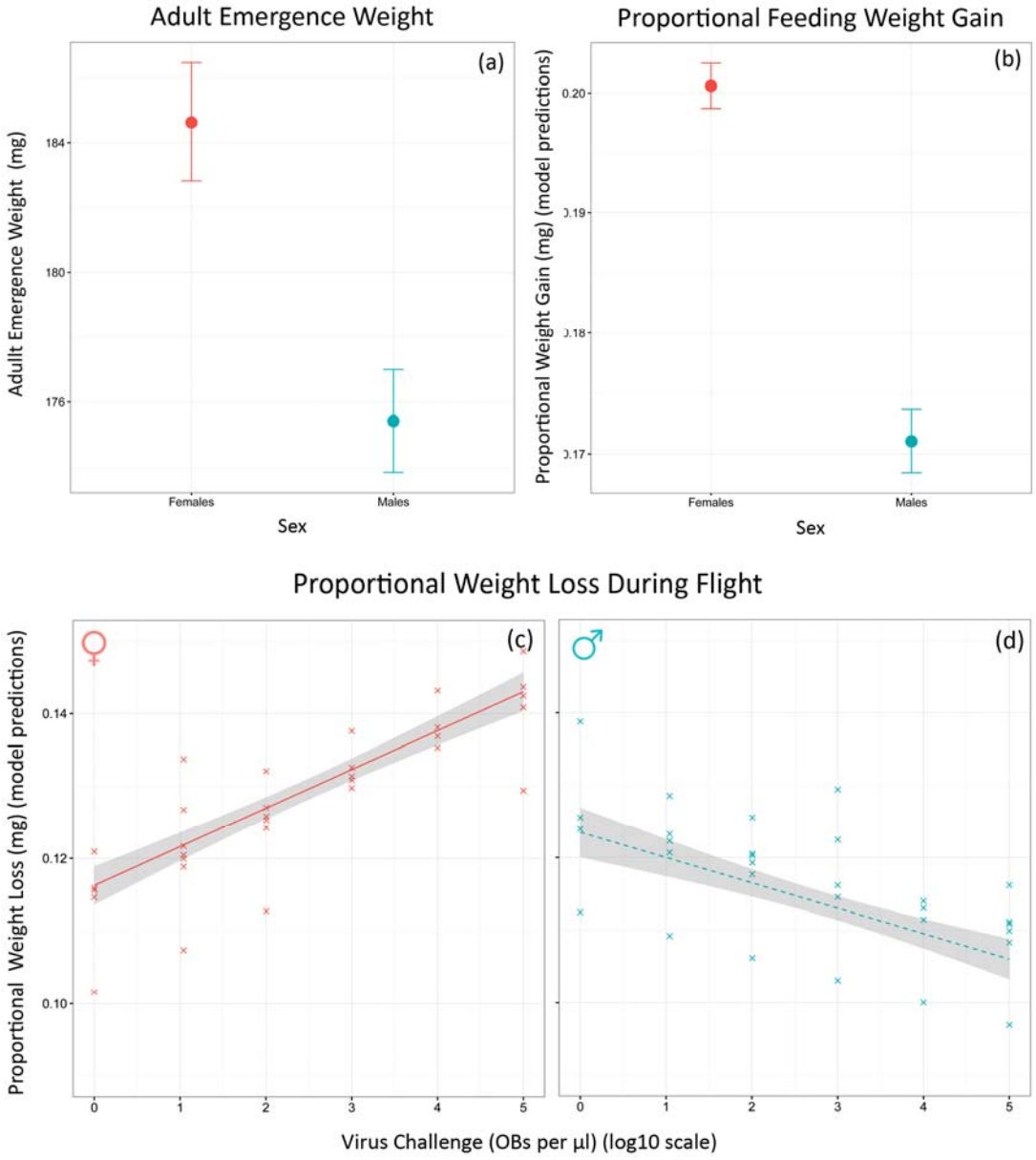

Figure 3.8: The effect of pathogen challenge and sex on weight. Although there was no significant difference in weight at the start of pupation (not shown), females weighed more than males on emergence (a). They also imbibed more sucrose solution before flight (b). During flight however, females lost proportionally more weight with increasing pathogen challenge (c), while there was no significant effect in males (d). This relationship was not significant in males (d). Statistics are given in section 3.3.5.4. Grey bands show 95% confidence intervals.

3.4.5 WING LENGTH

The final variable that was investigated in terms of its relationship with virus challenge and sex was wing length.

Firstly, to investigate if wing length was associated with increased flight capacity, it was modelled as a function of total distance flown. Insects with longer wings achieved greater distances (LM: $F_{1,84} = 5.25$, $p = 0.02$, Figure 3.9). However, the amount of variation explained by wing length was very small: 4.8%, suggesting that while wing length may correlate with flight distance, it is unlikely to be a very good predictor in this species. There was no significant relationship between wing length and average flight duration, number of flights or maximum flight speed ($p > 0.05$). As such, wing length was a very weak predictor of flight capacity.

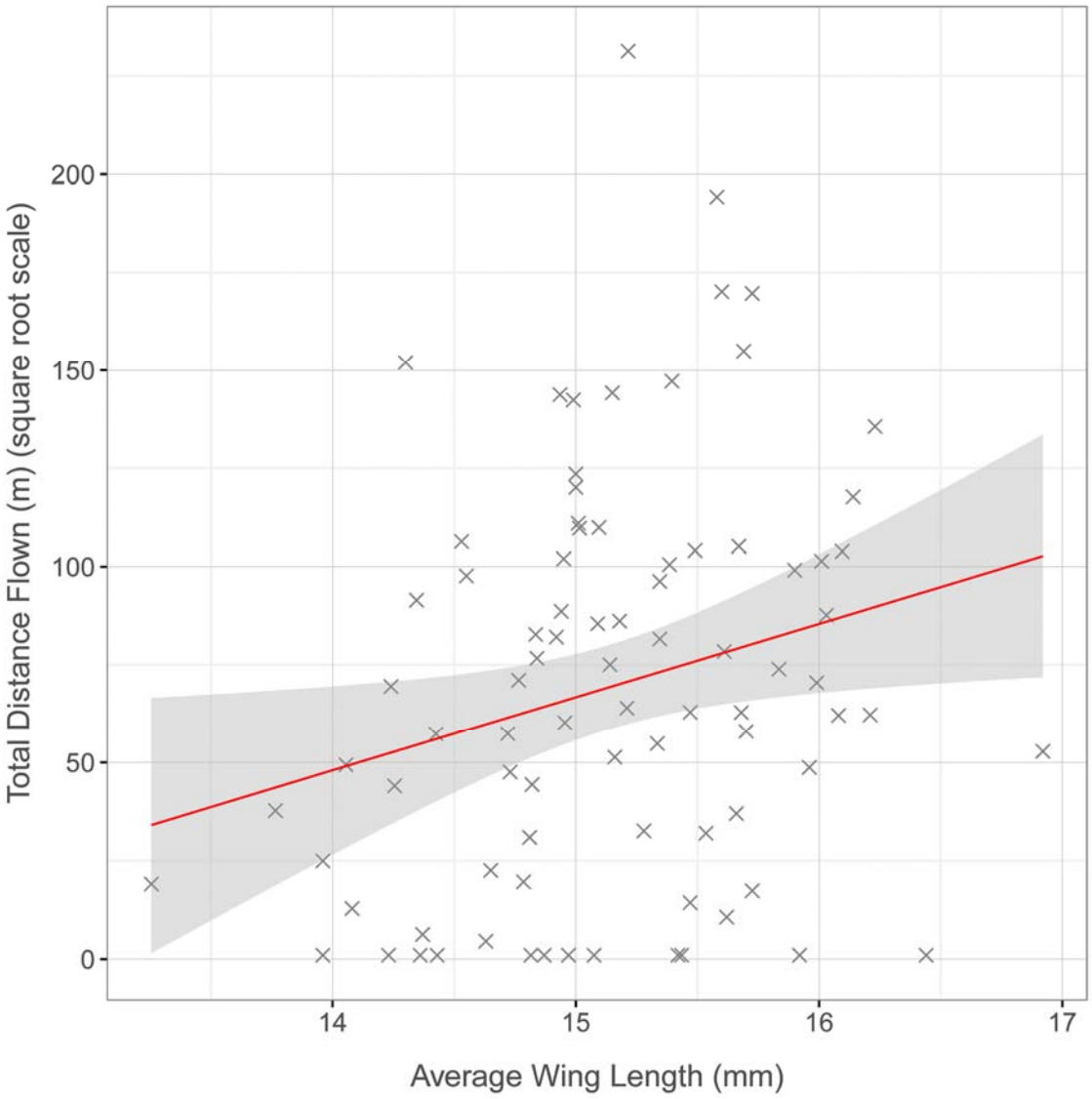

Figure 3.9: The relationship between wing length and total distance. Insects which had longer wings on average achieved greater total distances. However, this model only explains 4.77% of overall variation and as such suggests this is a relatively poor predictor of overall dispersal capacity in *S. frugiperda*. Statistics are contained in the text. Grey bands show 95% confidence intervals.

The second question was whether morphological variables related to flight, such as wing length, were affected by pathogen load or, alternatively, by factors such as development time, weight or sex which were shown to vary with wing length. In this case, if each of the development parameters was modelled individually then insects that had longer wings developed faster (LM: $F_{1,84} = 5.19$, $p = 0.030$, Figure 3.10a) and weighed more as pupae (LM: $F_{1,84} = 22.67$, $p < 0.001$, Figure 3.10b). Females also had longer wings on average than males (LM: $F_{1,84} = 14.59$, $p < 0.001$, Figure 3.10c). However, if all three terms (developmental rate, sex and pupa weight) were included in the same model, development time was no longer a significant predictor of wing length. This would suggest that, of the three terms, developmental rate is a poor predictor. This is supported by the fact that it explains only 4.7% of the variation in wing length. There was no effect of pathogen load in any of the models ($p > 0.05$).

Overall, these results would suggest that adult virus challenge has very little impact on wing length.

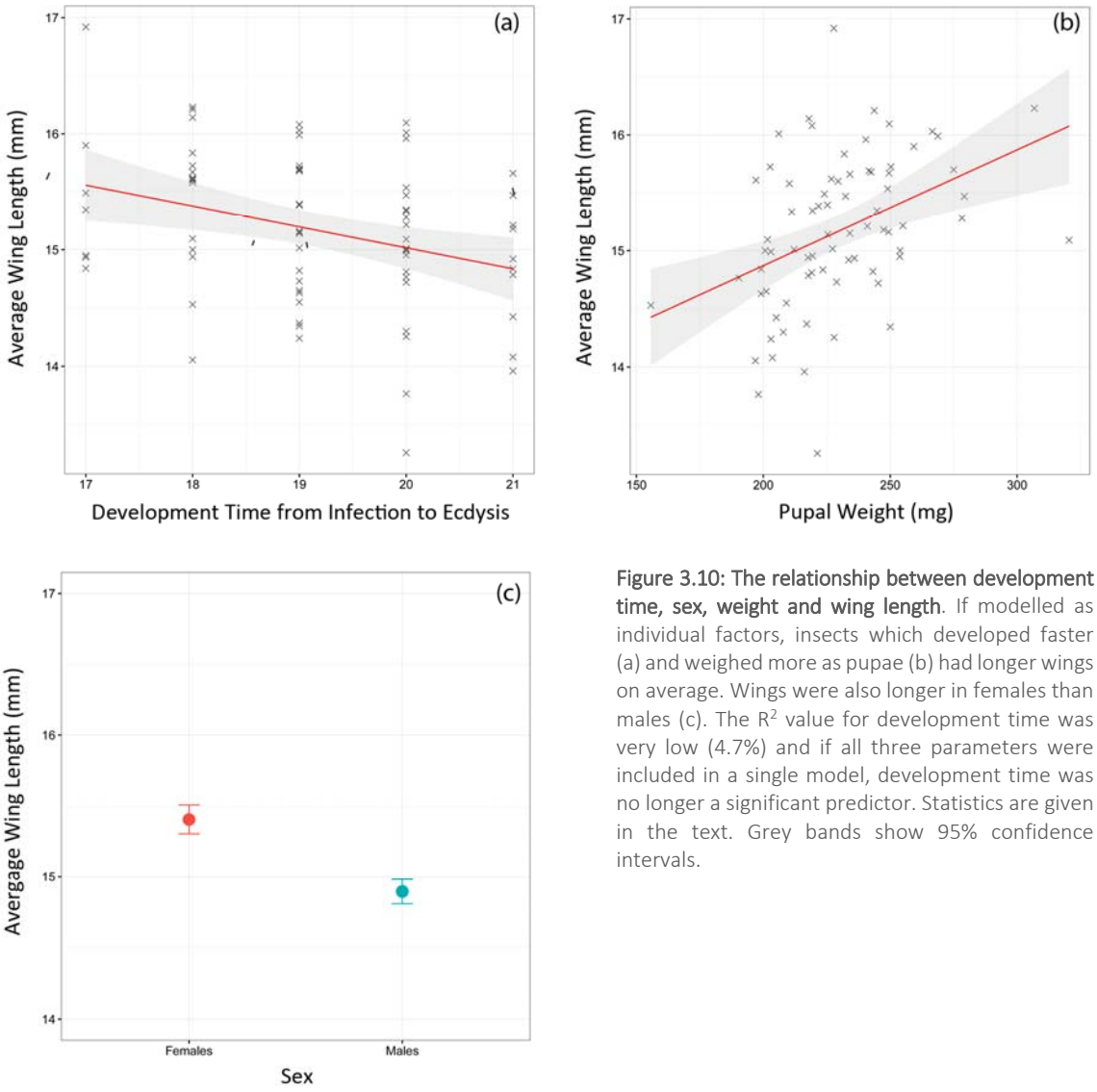

Figure 3.10: The relationship between development time, sex, weight and wing length. If modelled as individual factors, insects which developed faster (a) and weighed more as pupae (b) had longer wings on average. Wings were also longer in females than males (c). The R^2 value for development time was very low (4.7%) and if all three parameters were included in a single model, development time was no longer a significant predictor. Statistics are given in the text. Grey bands show 95% confidence intervals.

3.5 DISCUSSION

The key finding in this chapter is that, as a result of different life-history priorities, males and females have different migration-susceptibility cost functions, resulting in different forms of tolerance in migratory populations. In addition, the results show that differing cost functions are present throughout the infection cycle, and that these affect multiple aspects of the insects' response to virus exposure. To my knowledge, this is the first explicit demonstration of Bateman's principle affecting the life history traits that influence disease response and tolerance. These results are discussed first in terms of different cost functions in the two sexes, and how they are underscored by fundamental differences between males and females.

3.5.1 THE DIFFERING COST FUNCTIONS OF PATHOGEN CHALLENGE IN MALES AND FEMALES

While it has been proposed that pathogen challenge may affect flight capacity in multiple ways (Chapman et al., 2015b), the work here demonstrates that simply focusing on migratory capacity alone is not sufficient to explain the full implications of disease in migratory host populations. In this study, the results demonstrated significant statistical interactions between sex and pathogen challenge at multiple different points in the host's life cycle. Sublethal effects of virus challenge on developmental and physiological parameters have been observed in other baculovirus systems (Cabodevilla et al., 2011b). In the results presented here, virus challenge affected development time, flight behaviour (and hence flight capacity) and weight lost during flight differently in the two sexes. The nature of this relationship was such that females increased their development rate in response to increasing levels of pathogen challenge, but showed no change in their flight behaviour. Instead, increases in the amount of weight lost during flight in response to pathogen challenge would suggest females invested more resources in maintaining their flight capacity at higher levels of infection. In combination, these physiological and behavioural responses would seem to suggest females engage in a form of migratory escape from disease (Altizer et al., 2011) at multiple life-history stages.

In males, the response was fundamentally different. Males showed no change in their development time and maintained statistically similar amounts of weight loss during flight. Instead, males seemed to invest in a different flight strategy that resulted in decreases in flight capacity at higher levels of pathogen challenge. Specifically, this reduction in flight capacity was the result of changes in flight behaviour. In the control group, males undertook relatively few flights with the longest durations, resulting in the greatest total distances. At intermediate doses, the number of flights increased but the average duration decreased, leading to a decrease in

distance flown, and at the highest challenge doses both the number of flights and the average flight durations decreased again, resulting in the lowest overall total distances.

Being able to describe such behaviour using rotational flight mills is in itself novel (Chapter 2) and in relation to this study is particularly interesting as it suggests that males engage in behaviour that is fundamentally different to that of females and which would result in migratory culling rather than migratory escape (Altizer et al., 2011). This appears to be supported by results in the European corn borer, where the different cost functions between sexes were not explicitly defined but flight capacity of females appeared to be more resilient to infection with the microsporidian *Nosema pyrausta* than that of males (Dorhout et al., 2011, Villacide and Corley, 2008). In both the female corn borer and the woodwasp *Sirex noctilio* these changes in flight behaviour were accompanied by reductions in weight during flight that were greater in infected insects.

3.5.2 Differences in life history strategies between the sexes

In accordance with Bateman's principle (Bateman, 1948), the observed differences in tolerance between the two sexes should be underlined by significant differences in their life-history strategies (Rolff, 2002) and that is certainly the case here. In this study, those differences are demonstrated by the significant effect of sex and the interactions between sex and virus challenge, and this was observed for multiple different parameters. Females for example developed faster than males, weighed more at emergence and exhibited different flight behaviours, achieving greater total distances overall. This increase in flight effort may be attributable to the amount of sucrose solution females drank prior to flight, which was greater than that of males. This suggests females may have a different strategy to males when it comes to fuelling their flight. Further, it is interesting to observe that these differences occur at different points in the insect's life cycle. For example, there is no evidence of any significant difference between the sexes in weight or development time in the larval stage, but the fact that females were heavier at emergence might suggest that the underlying process which drives the differential response to pathogen challenge is occurring in the pupal stage and/or at adult emergence.

The prediction that virus-related changes in weight loss and development time would result in variation in morphology was not supported. In this respect, the results here only detected significant differences in wing length between the two sexes that were correlated with both development time and pupal weight, which may suggest there may be some indirect effects. However, these are likely to be weak, particularly as very little of the variation in wing length was explained by these two factors (approximately 20% for pupal weight and only 4.7% for

development time). Wing length was positively correlated with total distance flown but the interactions with sex and virus challenge were not significant and again the amount of variation explained was very small, suggesting that even though wing length differs in the two sexes, this difference is not enough to explain the difference in how the insects performed on the flight mills.

3.5.3 CONCLUSION

In line with Bateman's principal, this study has demonstrated that where underlying differences in the host's life history vary with sex, additional sex-biased differences in disease response can affect the cost functions associated with tolerance and susceptibility to disease in males and females. These different relationships could be assumed to affect the spatial spread of disease (Chapter 1), and while spatial and temporal synchrony between host and pathogen has been considered in the literature (Bauer et al., 2016), more work is needed to understand how differences in overlapping host populations of the same species might affect disease dynamics, particularly in relation to sex.

This work would also suggest that a more complete understanding of the costs of pathogen challenge on disease dynamics in migratory systems can be gained by investigating effects across the whole of an individual's life history. Schneider (2011), for example, describes how the response to infection can vary over the infection cycle and this is likely to be especially true of horizontal transmission in the baculoviruses, where infection can only occur in the larval stage but the disease process may still lead to sublethal effects at the adult stage.

CHAPTER 4.

MIGRATORY RESISTANCE: THE EFFECT OF FLIGHT EFFORT ON VIRUS LOAD

4.1 ABSTRACT

Host susceptibility and resistance are two key traits in determining the extent to which infection will affect an animal's ability to migrate. While many studies have investigated the extent to which disease limits migratory capacity ('migratory tolerance' of disease), few if any have attempted to assess the extent to which migratory effort affects resistance and hence pathogen load. Such studies are technically challenging as they require experimental manipulation of migratory effort but this information is essential if we are to understand the cost function of physical exertion in migratory host-pathogen systems.

To gain insights into the relationship between migratory effort and infection susceptibility, this chapter describes the use of rotational flight mills to experimentally manipulate flight effort in the fall armyworm *Spodoptera frugiperda* when infected with the baculovirus *S. frugiperda* multiple nucleopolyhedrovirus (*Sf*MNPV). Using a specifically designed qPCR protocol to measure virus load, two studies were designed to quantify the differential cost functions in host susceptibility given varying levels of pathogen challenge and flight effort.

In combination, these studies demonstrate that the amount of energy invested in flight can and does lead to increases in virus load, but the extent to which this occurs and the factors that influence it (sex and secondary infection) depend on the underlying nature of the system, including possibly the level of infection already present in the population. These results would suggest that differential responses to flight effort occur at different times in the migratory season and this is discussed in relation to the seasonal infection dynamics of migratory host-pathogen systems.

4.2 INTRODUCTION

Both migration and immunity are very costly processes (Dingle, 2014, Schmid Hempel, 2011), and the way in which these costs are balanced in migratory host-pathogen systems can impact macro-ecological processes such as population dynamics (Myers and Cory, 2016) and the geographic spread of disease (Altizer et al., 2011) (see also Chapter 1). The outcome of these relationships is ultimately driven by changes in the level of host susceptibility and pathogen virulence over time. In recent years, the cost of infection on migratory potential has been widely studied in a variety of systems (Morgan et al., 2007, Miller-Butterworth et al., 2014, Moore and Brown, 2014, Poulin et al., 2012) and this has led to the description of multiple possible mechanisms such as migratory culling and migratory escape (Altizer et al., 2011), as well as migratory spread of disease and migratory recovery (Shaw and Binning) (see also Chapter 1). These mechanisms have the potential to help us understand what drives changes in disease loads in migratory populations over time. Mass migratory movements, with associated costs (Bonte et al., 2012, Dingle, 2014) and benefits (Chapman et al., 2012) that they can incur for the hosts, must also have implications for the pathogen. Yet to date the literature on this subject has predominantly focused on the interaction between migration and disease from the perspective of the host, and the strategies pathogens might employ that enable them to adapt to or even take advantage of this movement have been less widely considered, although zoonotic disease diversity in migratory birds is something of an exception (see for example Latorre-Margalef et al., 2014, Runstadler et al., 2013).

One question that has not been directly addressed is how differences in the level of physical exertion affects the migratory resistance (the impact of behavioural, physiological or genetic adaptions linked with the migratory syndrome on pathogen load) and hence the host's level of susceptibility to infection. Testing this requires experimental manipulation of migratory effort, an approach that has yet to be tested in the literature but which has been theoretically considered by Chapman et al. (2015b) (Figure 1.4). Here the authors proposed that we might expect multiple different relationships to exist between migratory effort and host susceptibility depending on the underlying nature of the system. For example, with vector-borne diseases where disease transmission is expected to have very little cost for the vector (Schmid Hempel, 2011), we might expect there to be little or no cost between disease susceptibility and migratory effort. Alternatively, investment in immunity during migration (Arriero et al., 2015) might be assumed to reduce migratory resistance and hence host susceptibility. Conversely, the physiological cost of migration may lead to linear increases in resistance and hence susceptibility with increasing levels of migratory effort. These relationships may also be expected to vary within a system, for example where response to pathogen challenge differs between the sexes (Chapter 3) (Dorhout

et al., 2011)) or where different age groups have differing levels of susceptibility, as is known to occur in some migratory bird species (van Dijk et al., 2014).

While varying migratory capacity in a host-pathogen system is challenging, the use of rotational flight mills makes it possible to manipulate flight effort in migratory insects by forcing them to fly for a specified duration. By combining this approach with pathogen bioassays and quantitative polymerase chain reaction (qPCR), this chapter specifically sets out to undertake the first test of how effort invested in flight results in changes in pathogen load.

This relationship was quantified in the fall armyworm *Spodoptera frugiperda* when infected with the baculovirus *S. frugiperda* multiple nucleopolyhedrovirus (*Sf*MNPV). Given the results described in Chapter 3, it was specifically hypothesised that the relationship between flight effort and migratory resistance would vary between the sexes (Rolff, 2002) but also as a result of differing levels of pathogen challenge, which has been shown to affect virus load in the closely related *S. exigua* (Cabodevilla et al., 2011b). To investigate this relationship, two experiments were designed. The first, a five-hour forced flight trial (5hFF), manipulated the level of pathogen challenge and compared insects which were forced to fly for a continuous five-hour period with non-flown control groups. The second, a manipulated flight time trial (MFT), varied the amount of time insects were forced to fly and then compared the results between two pathogen treatments: a control and a challenged group (1,000 OBs per µl) which were dosed with the virus as third instar larvae. These experiments were used to test the following hypotheses:

5 hour forced flight (5hFF) trial:

The five-hour forced flight trial aimed to quantify if the level of pathogen exposure affected the migratory tolerance (disease load) in flown and unflown insects. The specific hypotheses tested were

i. Increasing levels of virus challenge will result in a linear increase in virus load (linear cost, figure 4.1) for all insects (Cabodevilla et al., 2011b), but the physical exertion associated with flight will result in lower levels of tolerance (higher pathogen loads; a steeper slope) in insects flown for five hours; and
ii. This relationship would vary between the sexes in line with Bateman's principle (Chapter 3).

Manipulated flight time (MFT) trial:

This experiment was designed to quantify the shape of the relationship between time spent flying and migratory tolerance. The specific hypotheses tested were

iii. Increasing flight durations will result in higher virus loads, as per hypothesis ii;
iv. Uninfected insects will show no change in virus load with increasing time spent flying ("no cost", figure 4.1) and remain statistically equivalent the unflown control group;
v. Infected insects will show a decrease in migratory tolerance (increase in pathogen load; linear cost in figure 4.1) with increases in the amount of time spent flying.
vi. This relationship will vary between the sexes in line with Bateman's principle (Chapter 3).

To assess replicability across these two experiments and compare results with Chapter 3, the same measurements of weight and development time were also taken wherever possible, leading to the following hypotheses:

vii. Increases in pathogen challenge will lead to decreases in development time in females ('migratory escape') but not males, although this might vary with life history stage (Cabodevilla et al., 2011b);
viii. Adult weight will decrease with increasing level of exposure to baculovirus infection during the larval stage (Cabodevilla et al., 2011b);
ix. Weight loss during flight would be higher in females than males, accounting for differences in immune response (Chapter 3)

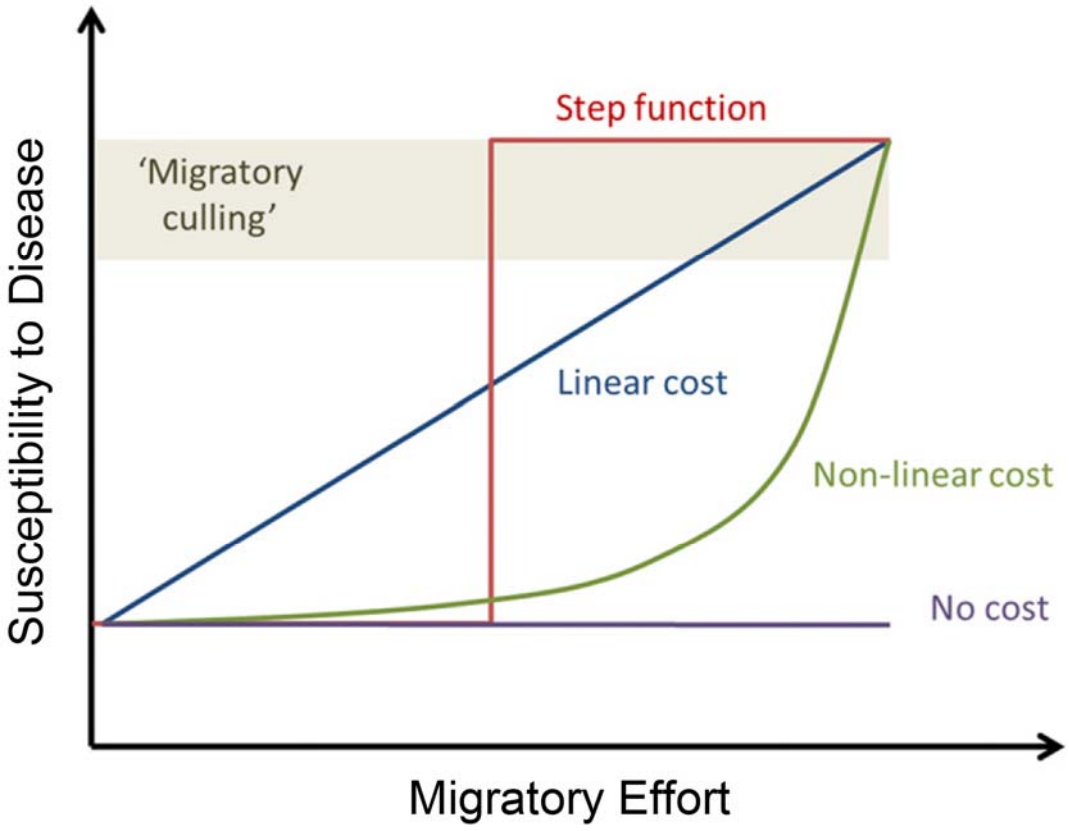

Figure 4.1: Theoretical cost functions for migratory resistance Migratory resistance (in the form of pathogen load and overall susceptibility to disease) is likely to depend on the effort an individual invests in migration, and may vary with resource investment for individuals which display a migratory phenotype. As the effort invested in migration increases, changes in susceptibility (in the form of either tolerance or resistance) might display different cost functions. This chapter takes two approaches to exploring the cost of flight effort on migratory tolerance in the fall armyworm when infected with the baculovirus *Sf*MNPV. The first approach looks at changes in the level of infection for a given flight duration depending on the level of pathogen exposure. The second approach considers the effect of time spent flying when the level of exposure is constant. Figure adapted from Chapman et al. (2015b)

4.3 METHODS

To reduce inbreeding and ensure experimental insects were within a few generations of field collected adults, two different fall armyworm cultures were used, one in each experiment. These came from field collections sampled in the same location at different times of year. In the 5hFF trial, larvae were collected in early summer (June 2015). In the MFT, larvae were collected later in the season, during the expected return migration period (October 2015). These two cultures were brought into the laboratory and reared using the same protocol (Appendix 2). The infection protocol, flight procedure and handling during both experiments were also. This is detailed fully below.

4.3.1 VIRUS CHALLENGE

The *Sf*MNPV stock used in Chapter 3 was amplified in whole insects by inoculating a long-term lab strain of *S. frugiperda* provided by the Universidad Pública de Navarra in Pamplona, Spain and purifying occlusion bodies (OBs) collected from cadavers with overt symptoms of baculovirus death in which the larval body tissue had been converted into OBs. Briefly, approximately 60 third instar larvae from the long-term lab strain were fed artificial diet that contained 10 µl of a 1:100 dilution of the virus stock used in Chapter 3. All cadavers with overt OB presence were collected in sterile micro-centrifuge tubes and homogenised in 0.1% SDS. Occlusion bodies were purified by centrifugation and re-suspension, transferred to a single vial and re-suspended in Milli-Q water, which was stored at -20˚C.

The resulting OB concentration was calculated using a Neubauer haemocytometer and the resulting concentration used to create a six level 10-fold series dilution of 10 to 1,000,000 OBs per µl, and distilled water as a control. The same baculovirus stock was used in both trials, although the standard curves for each trial were created separately.

Insects were monitored daily and on the day they became fourth instar they were randomly allocated to a treatment group and infected using the procedure described in Chapter 3.

Only insects which consumed all of the diet plug were used in experimental trials. Insect weight 24 hours post infection, date of pupation, pupal weight 24 hours after pupation and the day of adult emergence were recorded. Experimental insects were reared at 26 °C ± 2 °C with an 8h dark: 16h light regime, increases and decreases in light intensity simulating a 30-minute artificial dawn dusk period every day. A full description of the experimental design and methods for each experimental trial is given in sections 4.3.2 and 4.3.3.

4.3.2 FIVE-HOUR FORCED FLIGHT TRIAL (5HFF)

Fall armyworm larvae were collected from corn fields at the USDA ARS field site at College Station, Texas in June 2015 and shipped to Rothamsted Research as late instar larvae/pupae. Seventeen insects (5 females, 12 males) survived shipping. These were mated in a single Perspex cage, and provided with an *ad libitum* supply of sugar water (Appendix 2). Seventeen egg batches were collected and approximately two hundred larvae reared through to adulthood. Mortality was low in this first generation (F1) and there was no sign of overt virus. The insects used in this trial (F2) were collected as eggs and phase shifted within 24 hours of eggs being collected.

Three days after hatching, larvae were transferred to individual 37 ml pots lined with artificial diet (Appendix 2) and infected as described. Each treatment contained a minimum of sixty larvae, except for the highest treatment (1,000,000 OBs per µl) which had 24 individuals and was used only to check for 100% mortality (Table 4.1).

On the morning of emergence, the sex of each individual was determined. Within each treatment group, equal numbers of each sex were randomly assigned to one of two flight treatments: a non-flown control and insects which were forced to fly for a continuous five-hour period. The level of replication for each treatment is given in Table 4.1.

All insects were provided with cotton wool soaked in distilled water until approximately four hours before flight, when insects were prepared for attachment to the flight mills using the protocol in Chapter 2. Flown insects were placed on the flight mills at dusk. When the lights turned off, flown insects were forced to start flying by removing the paper platform, triggering an automatic flight response. The non-flown controls were left in individual 37ml pots and placed on top of the flight mills for the duration of the five-hour flight period.

Insects which stopped flying could be encouraged to continue by gently offering them a substrate to clasp with their tarsi and then removing it again. Insects which did not respond to this for a period of a minute or more were marked as flown to exhaustion and removed from the trial. Two banks of sixteen flight mills were used in this trial.

After the five-hour flight period, individuals were removed from the flight mills and placed with the non-flown controls. All insects were again provided with access to distilled water and then left in individual 37ml pots until the lights came on again, at which point they were weighed again so weight loss over the 24-hour period could be calculated, and then euthanised by freezing and storing them at -20˚C.

NPV Treatment	Insects infected (n)	qPCR replicates (n)		% NPV Mortality
		Flown	Not Flown	
Control	60	21	19	0.00%
10	56	12	18	0.00%
100	67	17	19	7.46%
1,000	64	12	11	32.81%
10,000	83	12	10	53.01%
100,000	114	1	2	94.74%
1,000,000	24	NA (all dead)		100.00%
Treatment total:	-	75	79	-
Total (n)	468	154		-

Table 4.1: Replication in the five-hour forced flight trial. Overview of the experimental design (two flown treatments over six virus treatments) with the levels of replication for the five-hour forced trial. Insects in the highest NPV treatment category were used only to assess 100% mortality and were not used in flight mill trials. Mortality is only related to NPV and does not include other causes of death (see Section 4.4.1)

4.3.3 MANIPULATED FLIGHT TIME TRIAL

In October 2015, fall armyworm larvae were again collected from corn fields at the USDA ARS farm at College Station, Texas and then shipped to Rothamsted Research as late instar larvae/ pupae. A starting population (F0) of ten females and ten males were used to create a lab culture, and insects were reared through two generations before being used in the trial (F3). Some evidence of low-level NPV infection was evident in this culture, including in the import generation.

Neonates were phase shifted on the day of hatching and transferred to individual containers at three days old. Larvae were monitored daily and infected using the described procedure on the day they were observed as fourth instar. In this trial, two different NPV treatments (an uninfected control and a pathogen challenge of 1,000 OBs per µl) were applied over five different flight treatments: insects which were frozen on emergence (FRZ), a non-flown control as per the five-hour forced flight trial (NF), and insects which were flown for 1, 3 or 5 hours (1H, 3H and 5H respectively). To assess overall mortality, a full bioassay was completed as part of the trial. An overview of the experimental design and replication in each treatment is given in Table 4.2.

On the morning that adults emerged from their pupal cases, individuals were sexed and equal numbers of each sex randomly allocated to the five flight treatments. All insects except those which were frozen on emergence were provided with cotton wool soaked in distilled water until the point where they were prepared for attachment to the flight mills and flown using the same procedure described in the five-hour forced flight trial.

Insects in this experiment were snap-frozen in liquid nitrogen and stored at -80˚C. Insect samples were crushed in a mortar and pestle using liquid nitrogen and a sub-sample used for qPCR reactions. Mortar and pestles were washed with a laboratory disinfectant, triple rinsed and sterilised under UV between samples.

NPV Treatment	Insects Infected (n)	qPCR replicates (n)					% NPV Mortality
		Frozen on Emergence (FRZ)	Not Flown (NF)	Flown (1H)	Flown (3H)	Flown (5H)	
Control	210	26	16	23	21	18	5.71%
10	40	-	-	-	-	-	2.50%
100	30	-	-	-	-	-	10.00%
1,000	226	17	14	16	20	18	33.63%
10,000	39	-	-	-	-	-	64.10%
100,000	40	-	-	-	-	-	85.00%
1,000,000	41	-	-	-	-	-	100.00%
Treatment total:	**-**	**43**	**30**	**39**	**41**	**36**	-
qPCR total (n)	**626**			**189**			-

Table 4.2: Replication in the manipulated flight time trial. Replication strategy and experimental design for the manipulated flight time trial, showing five flight treatments over two levels of pathogen transmission strategies, horizontal transmission (larvae – larvae, here a pathogen challenge of 1,000 OBs per µl) and vertical transmission (adult – offspring). Replication and mortality for the dose response that was run alongside the 10 NPV-flight treatments is also shown. The percentage mortality refers only to insects which died of overt baculovirus infection. Other causes of death are not included in this table, but can be found in section 4.4.1.

4.3.4 CALCULATING VIRAL LOAD

A real-time quantitative polymerase chain reaction (qPCR) protocol was developed following the methods of Graham et al. (2015) and is described here in accordance with the Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).

Insects were randomly allocated to qPCR processing batches, each batch containing a maximum of 25 samples and DNA extracted using two different methodologies. In the five-hour forced flight experiment DNA was extracted from whole insects by transferring them to a screw-cap micro-tube containing three 3 mm glass beads and 540 µl of phosphate buffered saline (PBS). The insects were crushed in a lysis buffer with a MG FastPrep-24™ 5G bead beater for 40 seconds at 5.0 m/sec. Insects from the manipulated time trial were crushed using liquid nitrogen (section 4.3.3). Whole insect DNA from both trials was extracted following the manufacturer's insect specific protocol for the DNEasy® Blood and Tissue kit (P/N 69506; QIAGEN®, UK) (Appendix 2). The purity of the DNA was assessed using a Nanodrop 2000c spectrophotometer (ThermoFisher Scientific, UK) and any replicates where the purity of the DNA had a 260:280 ratio below 1.6 were removed from the analysis. DNA was quantified using a Qubit® Fluorometer 2.0 (Invitrogen, UK) in conjunction with a Qubit® dsDNA BR Assay Kit (P/N: Q32853, Invitrogen, UK) per the manufacturer's instructions. After extraction, DNA was stored at -20°C.

To quantify covert viral load, the amplification of whole insect DNA was assessed against a standard curve (absolute quantification). The standard curve was constructed using the same DNEasy® Blood and Tissue kit to extract *Sf*MNPV DNA from the baculovirus stock used to infect the larvae. The purity of DNA was again assessed using a spectrophotometer and quantified using the Qubit® dsDNA HS Assay Kit (P/N: Q32851, Invitrogen, UK). Copy number was calculated with an online calculator (http://cels.uri.edu/gsc/cndna.html) assuming a genome size of 132,000 bp (Simon et al., 2011) (GenBank accession No. HM595733.1). The resulting stock was used to construct a standard curve with a 10-fold series dilution (range: 10 – 10,000,000 viral genomes).

The primers and hydrolysis probe used in the qPCR assay were designed by ThermoFisher Scientific, UK using their Custom TaqMan® Gene Expression Assay service (P/N 4332079) and are specific to the *Sf*MNPV *polyhedrin* gene found in the SfNIC (Simon et al., 2011). The forward primer 5'-CTTCCCTATCGTGAACGACCAA-3', reverse primer 5'-TGGGTCTCATGTTGATCACTAGGA-3' and hydrolysis TaqMan® probe 5'-6FAM-ACACGTCCATAATTTC-3' were used to amplify a 62 bp region of the *polyhedrin* gene.

The qPCR cycle was run on a Rotor-Gene Q (QIAGEN, UK). Each run included a maximum of 25 insect samples, the standard curve and a no-template control (NTC). Intra-assay variation was assessed with three technical replicates for the standard curve, NTC and all samples. For every reaction, 5 µl of whole insect DNA (RNase-free water for the NTC) was combined with 10 µl of TaqMan® Fast Advanced Master Mix (P/N 4444964; ThermoFisher Scientific, UK), 1 µl primer/probe assay mix and 4 µl RNase free water, giving a total reaction volume of 20 µl. The thermo-cycle programme was designed in accordance with the instructions for the TaqMan® Fast Advanced Master Mix: a two-minute incubation period at 50˚C, followed by a 20 second polymerase activation step at 95˚C and then forty cycles of 95˚C for 3 seconds and 60˚C for 30 seconds. Fluorescence data were acquired on the green channel from the FAM fluorophore covalently attached to the 5' end of the probe. The standard curve was analysed using the Rotor-Gene Q software (QIAGEN, 2016).

To account for variability between qPCR runs, insect samples were randomly allocated to processing batches, which were included as a random term in the mixed effects models. To standardise between processing batches, the quantification cycle (Cq) was standardised in all runs. Only those runs where the regression coefficient of determination (R^2) exceeded 0.99 and the PCR efficiency fell in the range of 90% - 110% were used in further analysis. Technical replicates of insect samples with a standard deviation above 0.5 were removed from the dataset (D'haene et al., 2010).

A full protocol for DNA extraction, virus purification and qPCR can be found in Appendix 2.

4.3.5 Statistics

The statistical modelling process used here is the same as that described in Chapter 2, with the exception that statistical models which fitted flight treatment as a five-level factor used the *anova()* command to iteratively drop terms based on likelihood ratio tests. As such results are reported with Chi-square (χ^2) rather than F statistics.

The statistical modelling in this section falls into three parts. The first part of the analysis investigates the effect of pathogen challenge on mortality in both experiments. To follow on from Chapter 3, I then investigate how pathogen challenge affects weight and development time in the two separate trials. The final analysis looks at virus load. To avoid over-parameterisation, virus load was modelled twice in each of the experiments. The first set of models investigates the interaction between pathogen challenge, sex and flight treatment, while the second set of models investigates how virus load varies as a result of different development and weight variables.

4.3.5.1 THE EFFECT OF VIRUS CHALLENGE ON MORTALITY

The effect of virus challenge on mortality was assessed using the same protocol described in Chapter 3. Briefly, a generalised linear model with a binomial error structure was used to correlate proportion mortality for all causes of death with virus challenge. The model used was:

> Logit (*proportional mortality due to a specific cause of death*) = α + β x *pathogen challenge* (log10 - transformed)

4.3.5.2 THE EFFECT OF VIRUS CHALLENGE ON WEIGHT AND DEVELOPMENT TIME

Weight and development variables were modelled using the same protocol described in Chapter 3, but some amendments were needed to take account of different experimental treatments. In this chapter insects were given an ad-libitum access to distilled water rather than being force fed sucrose solution, so the effect of pathogen challenge on weight gain prior to flight was not investigated. For post-flight weight and adult weight loss during flight it was also necessary to alter the models to include the different flight treatments. Random terms were the same as those described in Chapter 3.

Five hour forced flight trial:

The starting model for weight variables in this experiment looked at the three-way interaction between sex, virus challenge and flight treatment, considering larval weight post infection:

> *Weight variable* (mg) = α + β1 x *Virus challenge* (log10-transformed) + β2 x *Sex* + β3 x *Flight Treatment* + β4 x *Larval weight post infection* (mg) + β5 x *Virus challenge* (log10-transformed) x Sex + β6 x *Virus challenge* (log10-transformed) *x Flight Treatment* + β7 x *Sex* x *Flight treatment* + β8 x *Virus challenge* (log10-transformed) x *Sex* x *Flight Treatment*

Manipulated flight time trial:

Again, weight variables in this experiment were modelled as a function of the three-way interaction between flight treatment, virus challenge and sex, considering larval weight post infection. For post-flight weight and proportional weight loss due to flight, two starting models were used, one where flight treatment were modelled as a five-level factor ("factorial flight treatment" or FFT) and a second model where flight treatment was modelled as a continuous variable of 1, 3 and 5 hours ("time spent flying" or TSF):

Factorial flight treatment:

> *Weight variable* (mg) = α + β1 x *Virus challenge* (log10-transformed) + β2 x *Sex* + β3 x *FFT* + β4 x *Larval weight post infection* (mg) + β5 x V*irus challenge* (log10-transformed) x *Sex* + β6 x *Virus challenge* (log10-transformed) x *FFT* + β7 x *Sex* x *FFT* + β8 x *Virus challenge* (log10-transformed) x *Sex* x *FFT*

Time spent flying:

Weight variable (mg) = α + β1 x *virus challenge* (log10-transformed) + β2 x *Sex* + β3 x *TSF* + β4 x *Larval weight post infection* (mg) + β5 x *Virus challenge* (log10-transformed) x Sex + β6 x *Virus challenge* (log10-transformed) x *TSF* + β7 x *Sex* x *TSF* + β8 x V*irus challenge* (log10-transformed) x *Sex* x *TSF*

4.3.5.3 THE RELATIONSHIP BETWEEN VIRUS LOAD AND WEIGHT

Correlation coefficients were used to determine the level of inter-dependence between the four different weight variables (larval weight, pupal weight, pre- and post-flight weight). As post-flight weight correlated very closely with pre- and post-flight weight, these two terms were summarised by proportional weight loss during flight, as per Chapter 3. As the correlation, co-efficients between proportional weight loss, pupal weight and larval weight were all low (<0.4), these three variables were used for analysis.

To quantify resistance at different points in the insects' life history virus load proportional weight loss during flight and pupal weight were modelled as a function of the interaction between virus challenge, flight treatment and sex, considering larval weight at infection and total development time. This gave the starting model:

Virus load (log10-transformed) = α + β1 x *total development time* (days) + β2 x *larval weight* (mg) + β3 x *flight treatment* + β4 x *virus challenge* (log10-transformed) + β5 x *sex* + β6 x *pupa weight* (mg) + β7 *x proportional weight loss during flight* (mg) + β8 x *pupa weight* (mg) x *sex* + β9 x *pupa weight* (mg) x *virus challenge* (log10-transformed) + β10 x *pupa weight* (mg)x *flight treatment* + β11 x *proportional weight loss during flight* (mg) x *sex* + β12 x *proportional weight loss during flight* (mg) x *virus challenge* (log10-transformed) + β13 x *proportional weight loss during flight* (mg) x *flight treatment*

The model included the random terms day flown, sibling group, diet batch, set of 16 flight mills and the qPCR processing batch. As proportional weight loss during flight wasn't available for insects in the frozen within 12 hours of emergence (FRZ) in the MFT trial, this was modelled twice: once including the treatment FRZ but excluding proportional weight loss and its interactions, and again excluding the treatment FRZ but including proportional weight loss and its interactions.

4.3.5.4 BETWEEN VIRUS LOAD AND DEVELOPMENT

Variation in resistance was also investigated as a function of development time, in a stating model that considered the development time of two different life stages and their interactions with sex. The starting model included both larval and pupal development time, investigating interactions between sex, flight treatment and virus challenge and accounting for pupal weight:

Virus load (log10-transformed) = α + β1 x *flight treatment* +
β2 x *virus challenge* (log10-transformed) + β3 x *pupa weight* (mg) +
β4 x *larval development time* (days) + β5 x *pupal development time* (days) +
β6 x *larval development time* (days) x *Virus challenge* (log10-transformed) +
β7 x *larval development time* (days) x *sex* +
β8 x *larval development time* (days) x *flight treatment* +
β9 x *pupal development time* (days) x *Virus challenge* (log10-transformed) +
β10 x *pupal development time* (days) x *sex* +
β11 x *pupal development time* (days) x *flight treatment*

Random terms investigated were day flown, sibling group, diet batch, set of 16 flight mills and the qPCR processing batch.

4.3.5.5 THE EFFECT OF FLIGHT TREATMENT ON VIRUS LOAD

To quantify how flight effort affected viral replication and hence migratory resistance, virus load in both studies was modelled as a function of flight treatment, with a three-way interaction between flight treatment, sex and pathogen challenge. Total development time, larval and pupal weights were also accounted for, giving the starting model:

Virus load (log10-transformed) = α + β1 x *flight treatment* +
β2 x *virus challenge* (log10-transformed) + β3 x *sex* + β4 x *pupa weight* (mg) +
β5 x total development time (days) +
β6 x *flight treatment* x *virus challenge* (log10-transformed) +
β7 x *flight treatment* x *sex* + β8 x *virus challenge* (log10-transformed) x *sex* +
β9 x *flight treatment* x *sex* x *virus challenge* (log10-transformed)

Random terms investigated for all models were day flown, sibling group, diet batch, set of 16 flight mills and the qPCR processing batch.

4.4 RESULTS

The results here are presented in four sections. The first compares mortality between the two trials (section 4.4.1. The second and third examine the effect of pathogen challenge development (section 4.4.2) and weight (section 4.4.3) in both experiments, and quantifies how these relate to virus load. The final section looks at the effect of flight treatment on viral load (section 4.4.4).

4.4.1 DIFFERENCES IN MORTALITY BETWEEN TRIALS

In the five-hour forced flight trial a total of 609 insects were infected, of which 266 (43.7%) survived to adulthood and 202 (33.2%) succumbed to overt baculovirus infection. Other causes of death included insects which failed to successfully pupate (4.1%), died during pupation (13.3%) and insects which died during emergence (3.9%). Of these causes of death, only pathogen challenge correlated with NPV-related mortality (Binomial GLM: $Z = -11.06$, $p < 0.001$, Figure 4.2). There was no evidence of baculovirus-related mortality in the control group of this trial.

In contrast, there were virus-induced deaths in the control group of the second trial (5.7% of insects infected with distilled water, Figure 4.2). Overall, of the 746 insects infected, 434 survived to adulthood (58.2%) and 192 (25.6%) succumbed to overt virus infection. Other causes of death included two insects which failed to emerge as moths (0.3%) and 85 insects which died during pupation (11.4%). Ten insects died of unknown causes (1.4%). Of these various mortality factors, deaths related to overt baculovirus infection were most strongly correlated with pathogen challenge (Binomial GLM: $Z = -11.27$, $p < 0.001$, Figure 4.2). Pathogen challenge was also significantly correlated with unknown death causes (binomial GLM: $Z = -4.33$, $df = 6$, $p < 0.001$), with the proportional mortality increasing with pathogen challenge. As the 10 insects which died of unknown causes showed no sign of overt infection under a phase-contrast light microscope (Figure 1.1), they were removed from further analysis.

In summary, the causes of death are broadly comparable between the two trials. The only point of interest is that vertical transmission of the virus from adult to offspring leading to spontaneous outbreaks of infection in the control group in the manipulated flight trial. This was not observed in the five-hour forced flight trial.

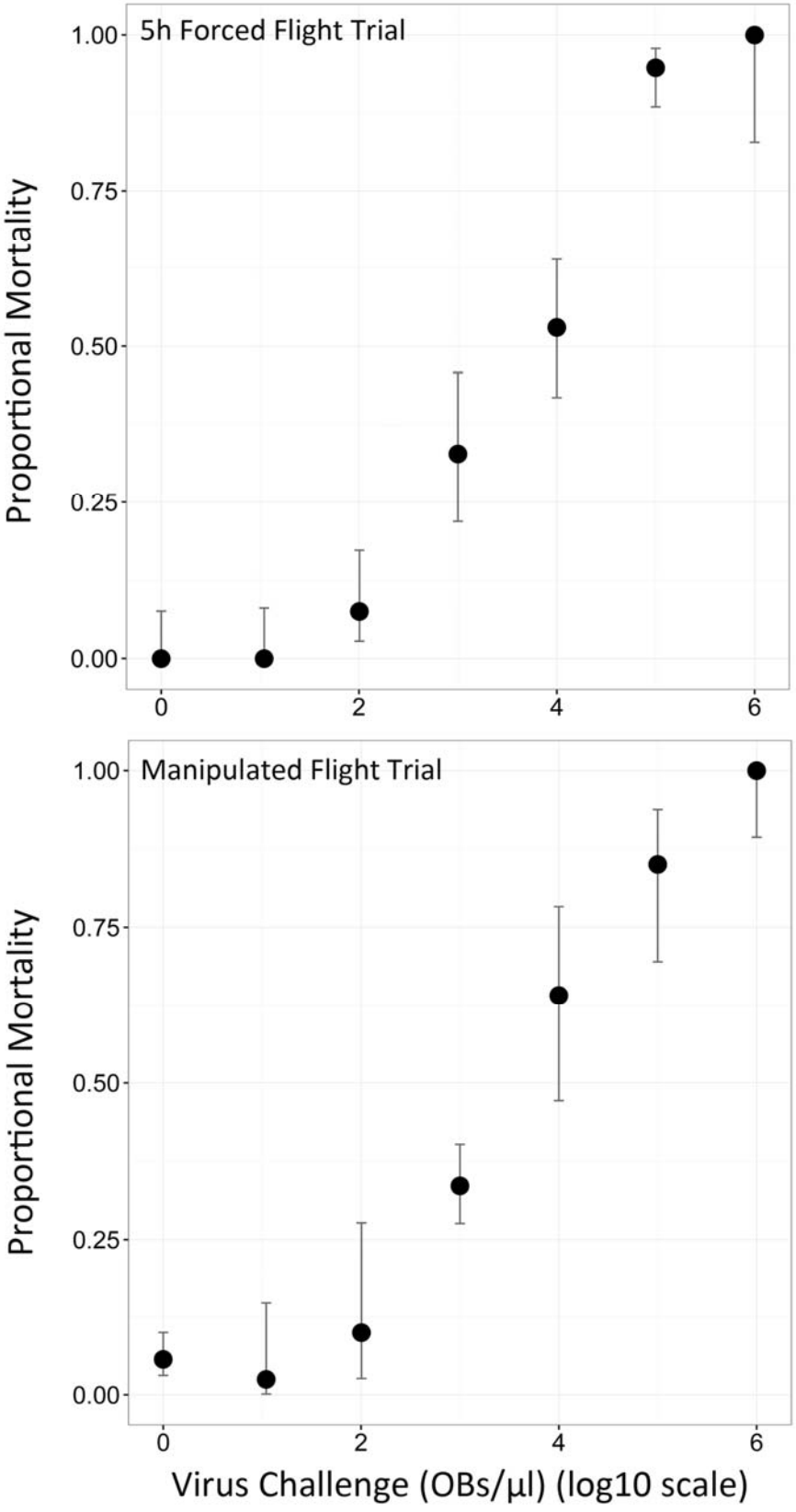

Figure 4.2: NPV-related mortality as a result of pathogen challenge. The proportion of insects succumbing to pathogen challenge in each of the six treatment groups for the five-hour forced flight trial (top) and the manipulated time trial (bottom). Error bars show binomial confidence intervals.

4.4.2 DEVELOPMENT

In both trials, the correlation between larval development rate and pupal development rate was very low (5hFF: Pearson's $r = 0.14$, MFT: $r = 0.05$), suggesting that there is very little association between the development rate of the two life history stages.

The effect of pathogen challenge on larval development time (LDT)

The only significant predictor of larval development time was larval weight at infection (5hFF LME with date infected, sibling group and diet batch as random terms: $F = 270.25$, $n = 276$, $p < 0.001$; MFT LME with sibling group as a random term: $F = 480.06$, $n = 418$, $p < 0.001$), with heavier larvae taking less time to reach pupation.

The effect of pathogen challenge on pupal development time (PDT)

In the five-hour forced flight trial, there was a significant interaction (LME with date infected and sibling group as random terms: $F = 5.21$, $n = 270$, $p = 0.023$, Figure 4.3) between sex and virus challenge, where development time was unaffected in females ($t_{118} = -1.67$, $p = 0.10$) but showed a significant increase with pathogen challenge in males ($t_{147} = 2.92$, $p = 0.004$). In addition to this interaction, there was also a significant difference between the sexes, with females developing faster than males on average (LME with date infected and sibling group as random terms: $F = 7.87$, $n = 270$, $p = 0.006$). Larval weight was also a significant predictor of PDT, with heavier larvae at infection developing faster than lighter larvae (LME with date infected and sibling group as random terms: $F = 74.96$, $n = 270$, $p < 0.001$)

In the manipulated flight time trial, neither larval weight nor the interaction between sex and virus challenge explained significant variation in the PDT ($p > 0.05$) but the difference between the sexes remained significant, with females again developing faster than males (LME with sibling group as a random term: $F = 409.73$, $n = 418$, $p < 0.001$; Figure 4.3).

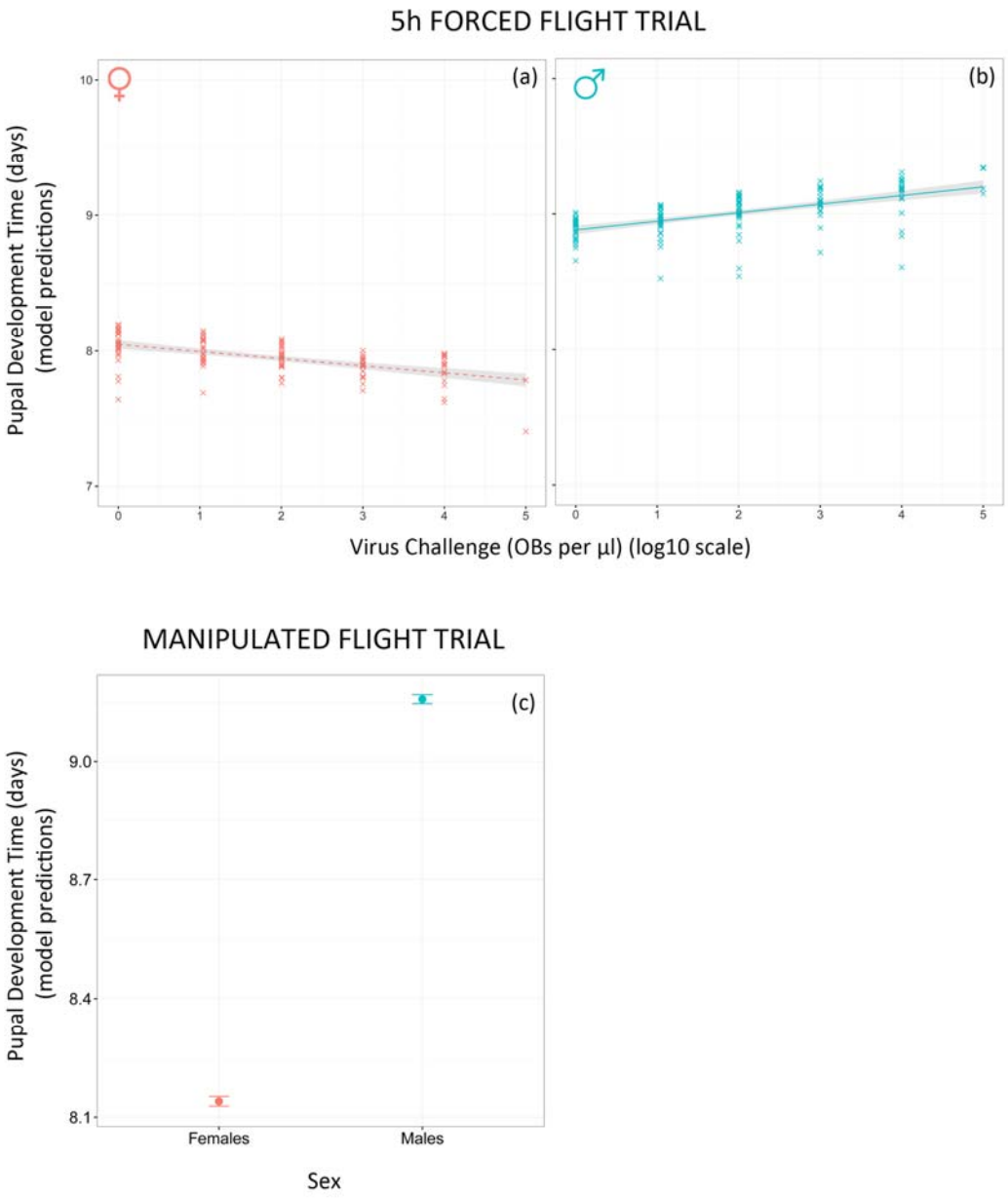

Figure 4.3: The relationship between virus challenge and pupal development time in two flight mill experiments. In both experiments, males took longer to develop as pupae than females (a – c). In the first experiment (5hFF) there was an additional interaction between sex and pathogen challenge, where female development time did not vary with virus challenge (a) but male development time increased significantly with increasing exposure (b). This interaction was not significant in the manipulated flight trial. Statistics are given in the text and full parameter estimates are presented in Appendix 5. Grey bands and error bars show 95% confidence intervals.

The effect of pathogen challenge on total development time (TDT)

When the development times of the two life history stages were combined, there was a significant difference in development time between the two sexes in both trials (5hFF LME with date infected, sibling group and diet batch as random terms: $F = 132.42$, $n = 270$, $p < 0.001$; MFT LME with date infected as a random term: $F = 164.88$, $n = 418$, $p < 0.001$), with females developing significantly faster on average. Larval weight was also a significant predictor in both experiments, with heavier larvae developing faster on average (5hFF LME with date infected, sibling group and diet batch as random terms: $F = 199.29$, $n = 270$, $p < 0.001$; MFT LME with date infected as a random term: $F = 447.02$, $n = 418$, $p < 0.001$).

Pupal weight, however, was only a significant predictor in the manipulated time trial. Interestingly, where this relationship was negative for larval weight at infection, pupal weight was positively correlated with development time, with heavier pupae taking longer to develop on average (LME with date infected as a random term: $F = 17.85$, $n = 270$, $p < 0.001$, Figure 4.4).

The relationship between development time and virus load in adult moths

The development parameters were only significant predictors of virus load in the five-hour forced flight trial. In this experiment, pupae that took longer to develop had higher pathogen loads (LME with processing batch as a random term: $F = 5.12$, $n = 156$, $p = 0.027$, Figure 4.4a). There was also a significant interaction ($F = 4.35$, $n = 156$, $p = 0.039$, Figure 4.4) between larval development time and sex, where there was no effect of larval development time on virus load in females ($t_{70} = -0.93$. $p = 0.355$, Figure 4.4a) while increases in development time in larvae lead to higher viral loads in males ($t_{82} = 2.01$, $p = 0.047$, Figure 4.4b). Sex ($F = 6.05$, $n = 156$, $p = 0.046$), flight treatment ($F = 9.52$, $n = 156$, $p = 0.003$) and pupal weight ($F = 7.83$, $n = 156$, $p = 0.0006$) were also significant terms in this model.

In the manipulated time trial, there was no effect of development time in any of the models of virus load ($p > 0.05$).

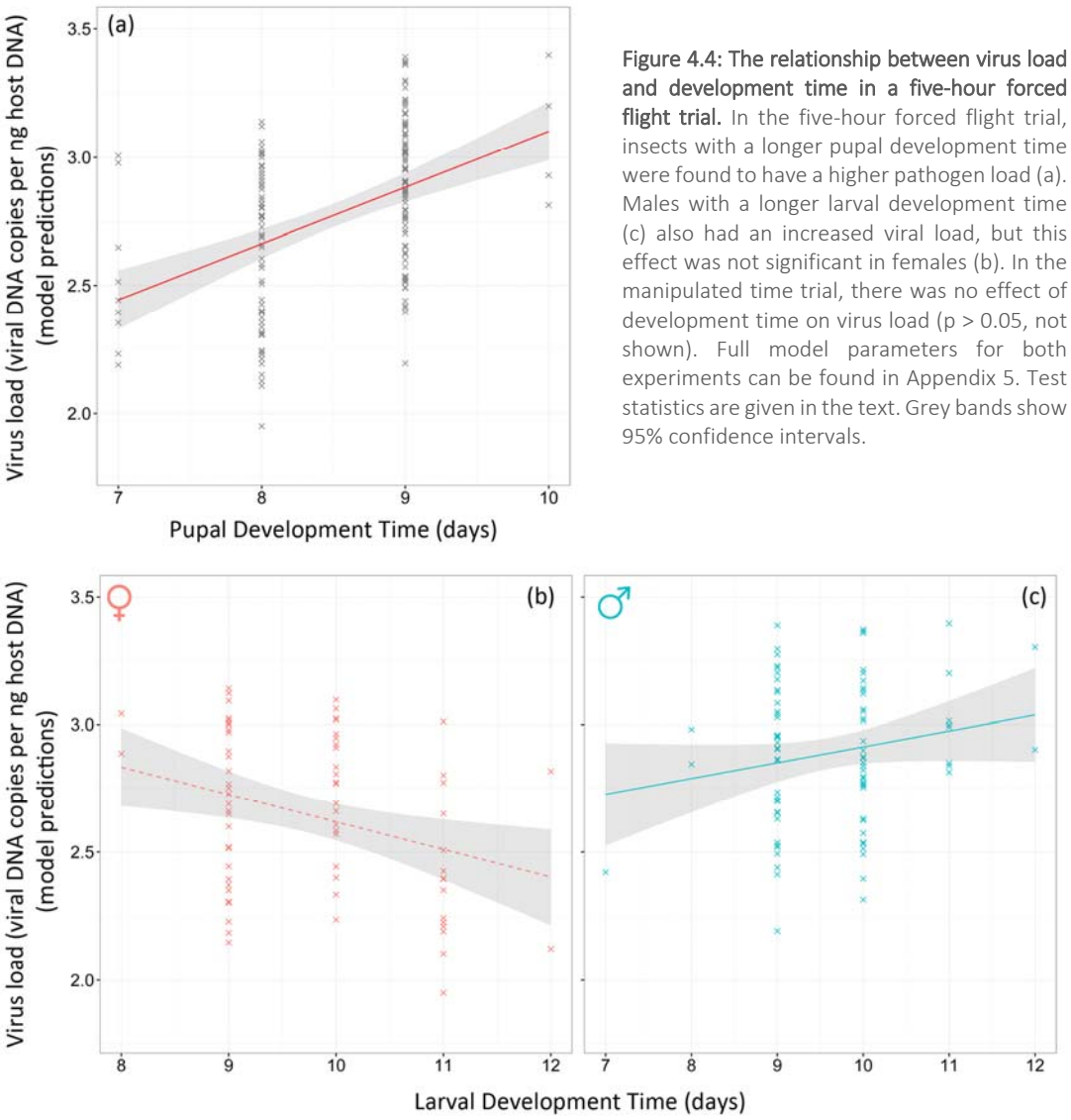

Figure 4.4: The relationship between virus load and development time in a five-hour forced flight trial. In the five-hour forced flight trial, insects with a longer pupal development time were found to have a higher pathogen load (a). Males with a longer larval development time (c) also had an increased viral load, but this effect was not significant in females (b). In the manipulated time trial, there was no effect of development time on virus load ($p > 0.05$, not shown). Full model parameters for both experiments can be found in Appendix 5. Test statistics are given in the text. Grey bands show 95% confidence intervals.

Summary

The factors which affected development time differed across the two different life history stages (larvae and pupae), and this in conjunction with the weak correlation between larval and pupal development times, would suggest that the response to virus exposure differs significantly in these two life stages (also observed in Chapter 3).

There are also differences between the two experiments. In the five-hour flight trial, virus challenge had a very clear effect on pupal development time, with marked differences between the two sexes. In the manipulated flight time trial, virus challenge was a very weak predictor on pupal development time (largely reliant on a few influential observations). However, in both experiments, males took longer to develop than females (Figure 4.3), a result which seems to be consistent as this is comparable with what was observed in Chapter 3.

Given these differences between the two experiments, it is perhaps unsurprising that development time was only correlated with virus load in the five-hour forced-flight trial, where once again the relationship differed between the two life-history stages.

4.4.3 PUPAL AND ADULT WEIGHTS

The effect of virus challenge and sex on pupal weight

Neither sex nor virus challenge were significant predictors of pupal weight in either of the experiments ($p > 0.05$). In the five-hour forced flight trial there were no significant predictors for pupal weight ($p > 0.05$) and in the manipulated flight time trial only larval weight was a significant predictor, with heavier larvae weighing more as pupae (LME with sibling group as a random term: $F = 9.19$, $n = 418$, $p = 0.003$).

The effect of virus challenge and sex on adult emergence weight

There was a significant interaction (LME with date infected and sibling group as a random term: $F = 5.44$, $n = 211$, $p = 0.02$) between pathogen challenge and sex in the five-hour forced flight trial, with males showing a decrease in adult emergence weight with increasing levels of pathogen challenge ($t_{113} = -2.39$, $p = 0.018$, Figure 4.5b). This relationship was not evident in females ($t_{71} = -1.98$, $p = 0.14$, Figure 4.5a).

In the manipulated flight trial, both sex and pathogen challenge were significant predictors of adult weight at emergence, such that infected adults weighed less at emergence than those in the control group (LME with sibling group as a random term: $F = 4.58$, $n = 140$, $p = 0.34$) and females weighed more than males (LME: $F = 24.36$, $n = 140$, $p < 0.001$). However, the effect of pathogen challenge was not significant ($p > 0.05$) if two influential observations were removed from the dataset - a female with a pre-flight weight of 215 mg and a male with a pre-flight weight of 242 mg, both in the control group. Sex remained a significant term when these outliers were included in the model (LME: $F = 31.53$, $n = 138$, $p < 0.001$) and as such is shown in Figure 4.5.

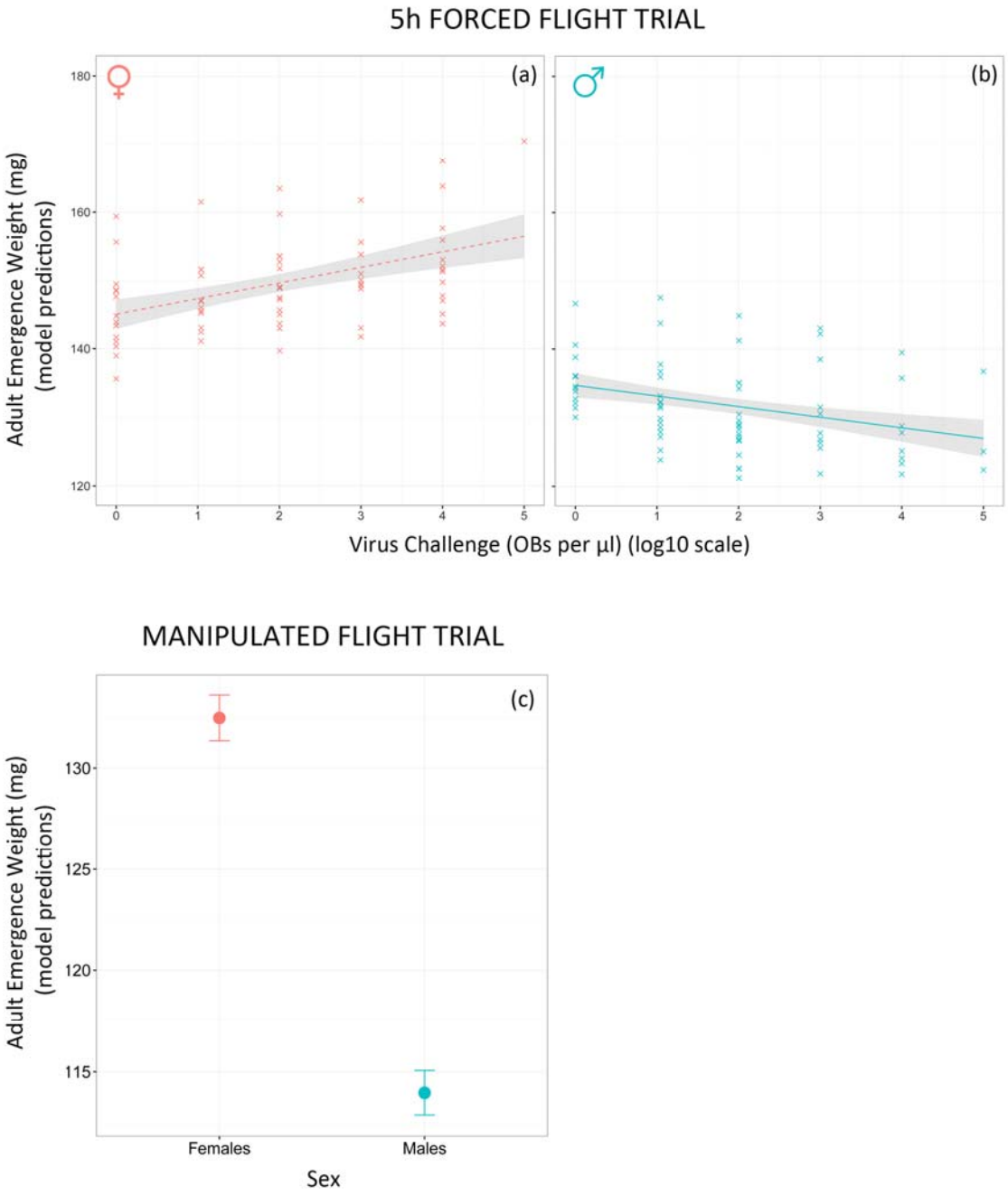

Figure 4.5: The relationship between virus challenge and adult emergence weight in two flight mill experiments. In both the 5hFF and MFT trials, adult emergence weight was lower in males (a – c) despite pupae of both sexes weighing equivalent amounts. In the 5hFF trial, there was also a significant interaction between sex and virus challenge, such that adult emergence weight did not vary significantly in females (a) but decreased with increasing pathogen challenge in males (b). In the MFT trial, there was also evidence that virus challenge resulted in lower adult weights (not shown) but this was not different among the sexes and became non-significant when several outliers were removed (see text). Full parameters estimates are given in the text and in Appendix 5. Grey bands and error bars show 95% confidence intervals.

The effect of sex, virus challenge and flight treatment on proportional weight loss during flight

As is to be expected, flight treatments had a significant effect on proportional weight loss during flight. In the five-hour forced flight trial, flown insects lost more weight than non-flown insects (LME with diet batch as a random term: $F = 4.42$, $n = 180$, $p = 0.037$) and males lost more weight than females (LME: $F = 41.12$, $n = 180$, $p < 0.001$; Females 9.16% ± 0.21%, Males 10.93% ± 0.20%).

In the manipulated flight time trial, increases in time spent flying also resulted in greater proportional weight loss (LME with diet batch as a random term: $F = 22.67$, $n = 110$, $p < 0.001$) and again males lost more weight than females (LME: $F = 7.45$, $n = 110$, $p = 0.007$; Females 9.98% ± 0.28%, Males 10.79% ± 0.19%).

The relationship between pupal weight and virus load

In both experiments pupal weight was significantly correlated with virus load, such that moths with higher viral loads weighed less as pupae (5hFF LME with processing batch as a random term: $F = 6.4$, $n = 153$, $p = 0.013$, MFT LME with processing batch and flight mill bank as random terms: χ^2: 4.40, $n = 139$, $p = 0.036$, Figure 4.6). However, in the five-hour forced flight trial this relationship was largely driven by outliers: a non-flown male with a pupal weight over 350mg which received 100 OBs per µl; a flown female which received 10 OBs per µl and had a weight over 350mg; and two non-flown males, one which received 100 OBs per µl and the other 1,000 OBs per µl. If these outliers were removed, the relationship between pupal weight and virus load was no longer significant in the 5hFF trial (see Appendix 5)

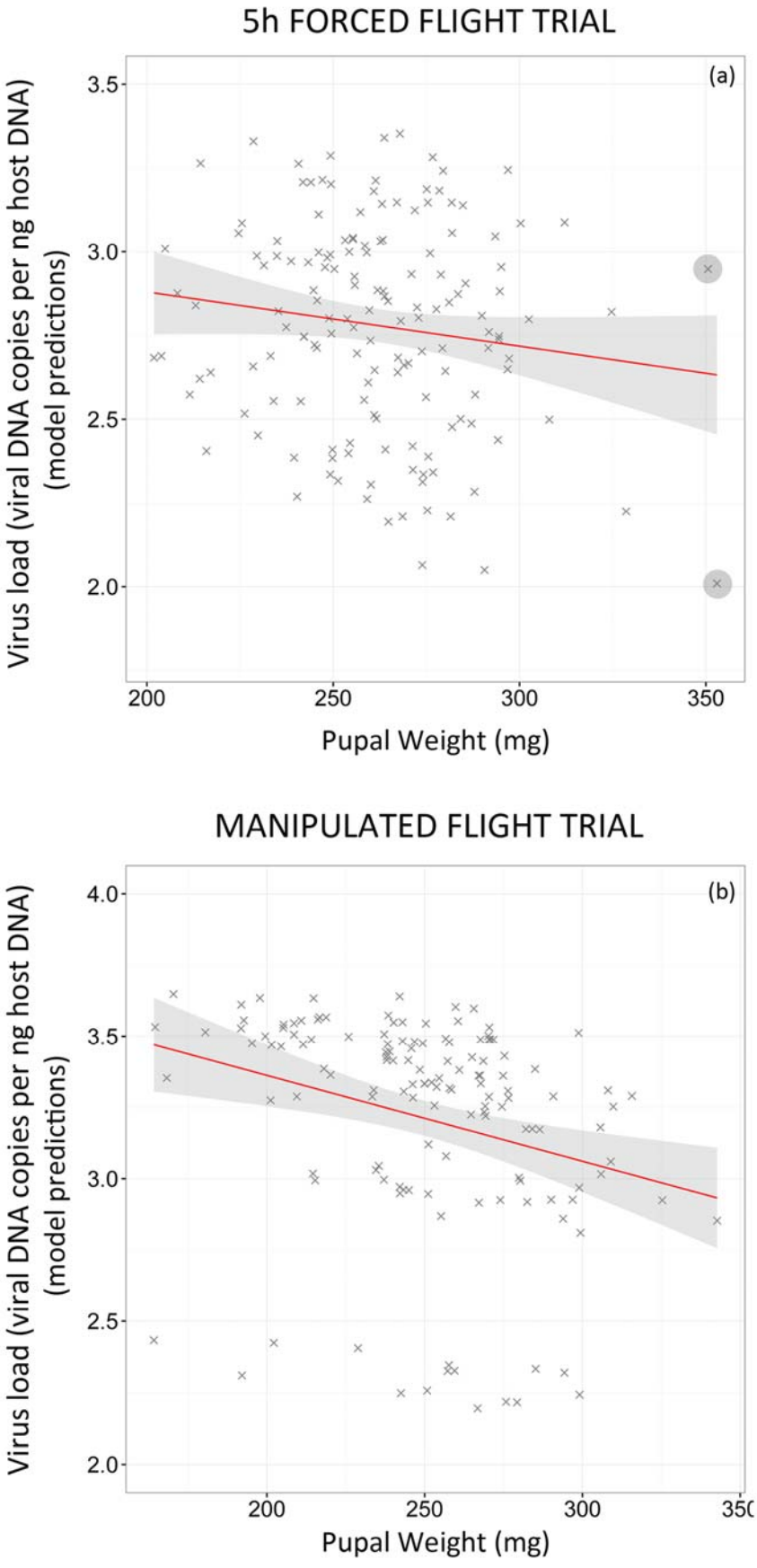

Figure 4.6: The relationship between pupal weight and virus load in two flight mill experiments. In both experiments, pupal weight was negatively correlated with virus load in adult moths (a,b). However, in the 5hFF trial, this relationship was reliant on two outliers (grey circles in a), without which there was no relationship between the two variables. Full statistics are given in the text and parameters estimates are available in Appendix 5.Grey bands show 95% confidence intervals.

Summary

The effect of virus challenge on weight was shown to vary depending on the life history stage at which the insect was measured, affecting adult emergence weight but not pupal weight. This is also true for the effect of sex, which only described changes in weight at adult emergence and during flight, but did not differ with sex when the insects are measured 24 hours after pupation.

Again, there were also differences between the two experiments. While sex affects both pupal weight and weight loss during flight in both experiments, the effect of pathogen challenge is only evident in the five-hour forced-flight trial (Figure 4.5). These results are broadly comparable with observations in Chapter 3.

In addition, the relationship between weight and virus load also varied between the two experiments. In both cases, an increase? In pupal weight was correlated with a decrease in virus load, but in the five-hour forced flight trial this was heavily dependent on outliers and influential observations, without which there was no significant correlation (Figure 4.6).

4.4.4 FLIGHT

Five hour forced-flight trial

In the five hour forced flight trial, flight lead to a significant increase in pathogen load (LME with processing batch as a random term: F = 8.9, n = 156, p = 0.003, Figure 4.7) and there was also a significant difference between the sexes, with males having higher viral loads than females (LME: F = 10.95, n = 156, p = 0.001, Figure 4.7). There was no effect of pathogen challenge on viral load, but pupal weight was a significant term in this model (LME: F = 4.13, n = 156, p = 0.044).

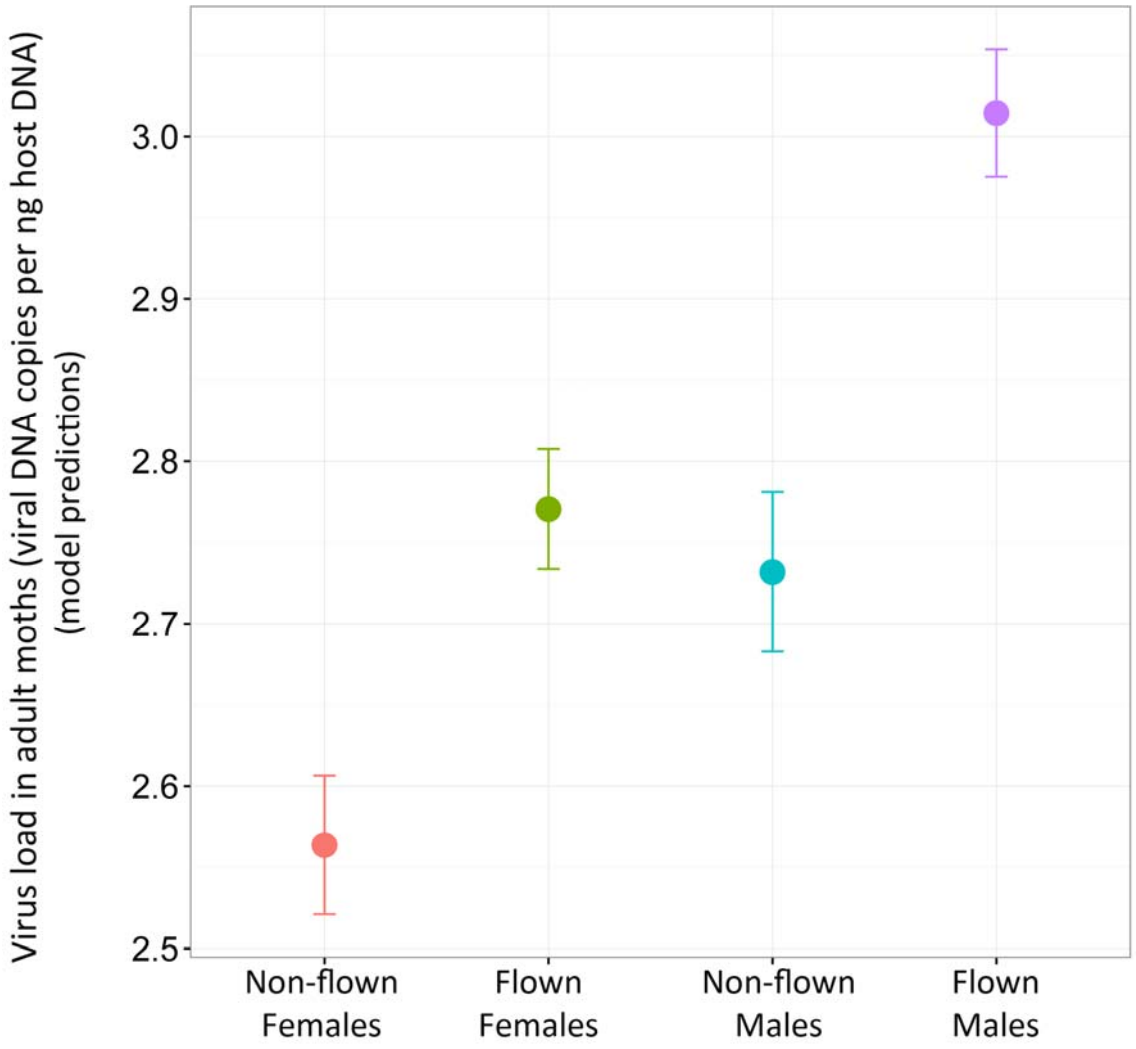

Figure 4.7: The effect of flight treatment and sex on virus load in a five-hour forced flight trial: In the 5hFF trial, there was a significant difference between the sexes and between insects which were forced to fly for five hours and those which were not flown at all. These differences were such that females had lower virus loads than males, but flight lead to an increase in virus load in both sexes. Full statistics are given in the text and parameter estimates are available in Appendix 5. Error bars show 95% confidence intervals.

In the manipulated flight time trial, the opposite was true. Sex was not a significant term in the model ($p > 0.05$) and there was no evidence of a change in virus load as a result flight (Figure 4.8). Instead virus load was described by a significant interaction between virus challenge (infected or control) and the five flight treatments (LME with processing batch as a random term: $\chi^2 = 9.95$, n = 175, $p = 0.041$), but this interaction was such that this appeared to be an age effect rather than a result of the amount of time spent flying. Specifically, insects in the control group which were flown for three hours, five hours or not flown at all within a 24-hour period had significantly lower virus loads than insects which were frozen within 12 hours of emergence (*3H – FRZ:* $t_{154.2} = -3.856$, $p = 0.002$; *5H – FRZ:* $t_{154.16} = -4.011$, $p = 0.009$; *FRZ – NF*: $t_{154.28} = 3.136$, $p = 0.017$, Figure 2.7). As such, the observed differences in viral load in the different treatment groups appears to be related to adult age rather than the amount of time in flight. This age effect was not observed in insects which had been challenged with the virus, in which instance all flight treatments were statistically similar.

To test this age effect further, the same statistical model was used to asses:

a) If there were differences in virus load between the three flight treatments (flown for 1h, 3h or 5h with a 24-hour period), which would suggest a flight-time effect.
b) If there were differences between insects frozen within 12h of emergence, insects flown for 5h and frozen after a 24-hour period, and insects which were non-flown and frozen after a 24-hour period, allowing age and flight effects to be distinguished.

The outcome of this analysis showed that there was no significant effect of time spent flying (1, 3 or 5 hours) on virus load in either of the virus treatments (control or virus challenged) ($p > 0.05$, Figure 4.8). However, there was a significant interaction between virus treatment and flight treatment (LME with processing batch as a random term: $\chi^2 = 11.15$, n = 100, $p = 0.004$). Once again, there was no difference between treatments in insects challenged with the virus ($p > 0.05$, Figure 4.9) but insects in the control group which had been kept alive for 24-hours had significantly lower virus loads than those which had been frozen within 12 hours of emergence, regardless of whether or not they had been flown (*FRZ-5H*: $t_{83.74} = -4.31$, $p = 0.001$; *FRZ-NF:* $t_{83.13} = 3.41$, $p = 0.003$; *5H – NF*: $t_{83.85} = -0.69$, $p > 0.05$; Figure 4.9), indicating that virus loads decline with moth age.

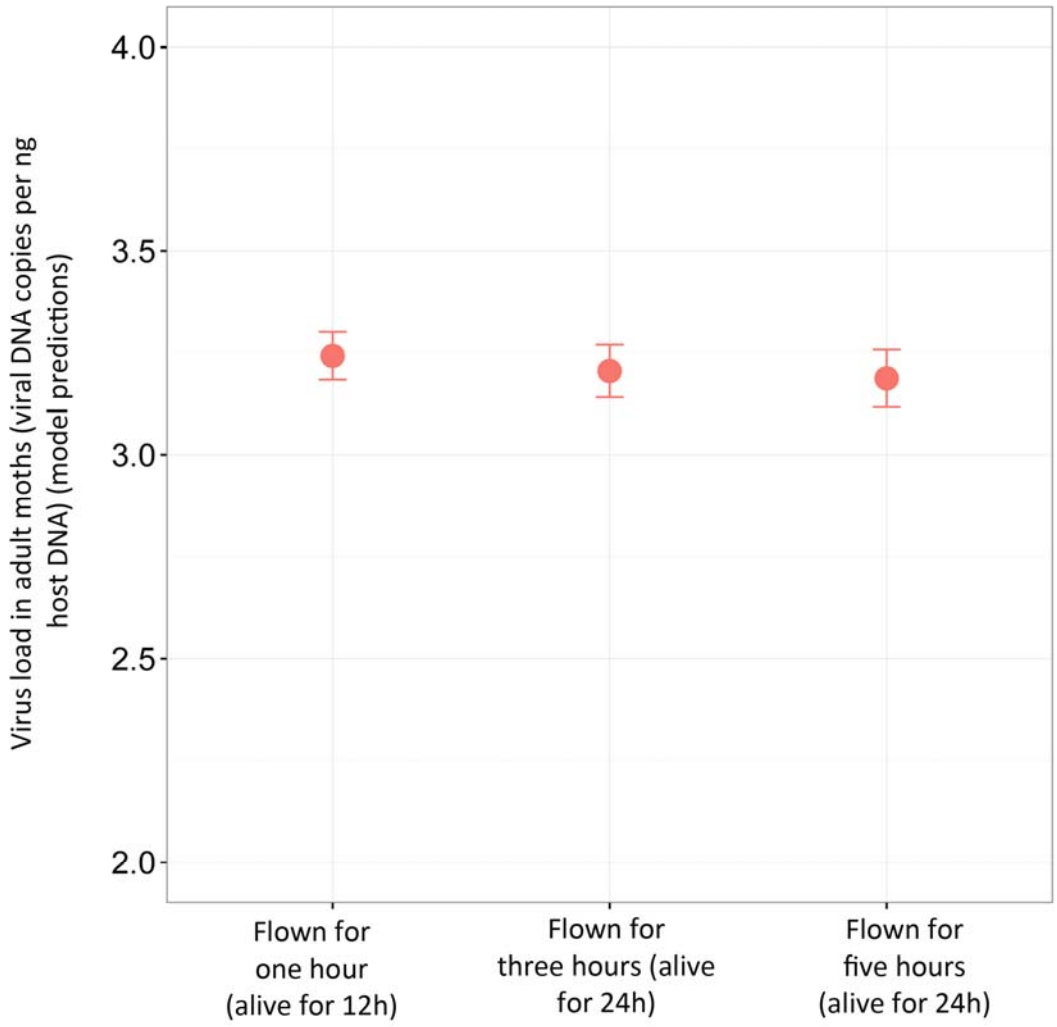

Figure 4.8: The effect of flight duration on virus load in the manipulated flight time trial (MFT). In this study, increases in the amount of time insects spent flying did not result in significant changes in virus load. Full statistics are given in the text and parameter estimates for development time (the only significant term in this model) are given in Appendix 5. Error bars show 95% confidence intervals.

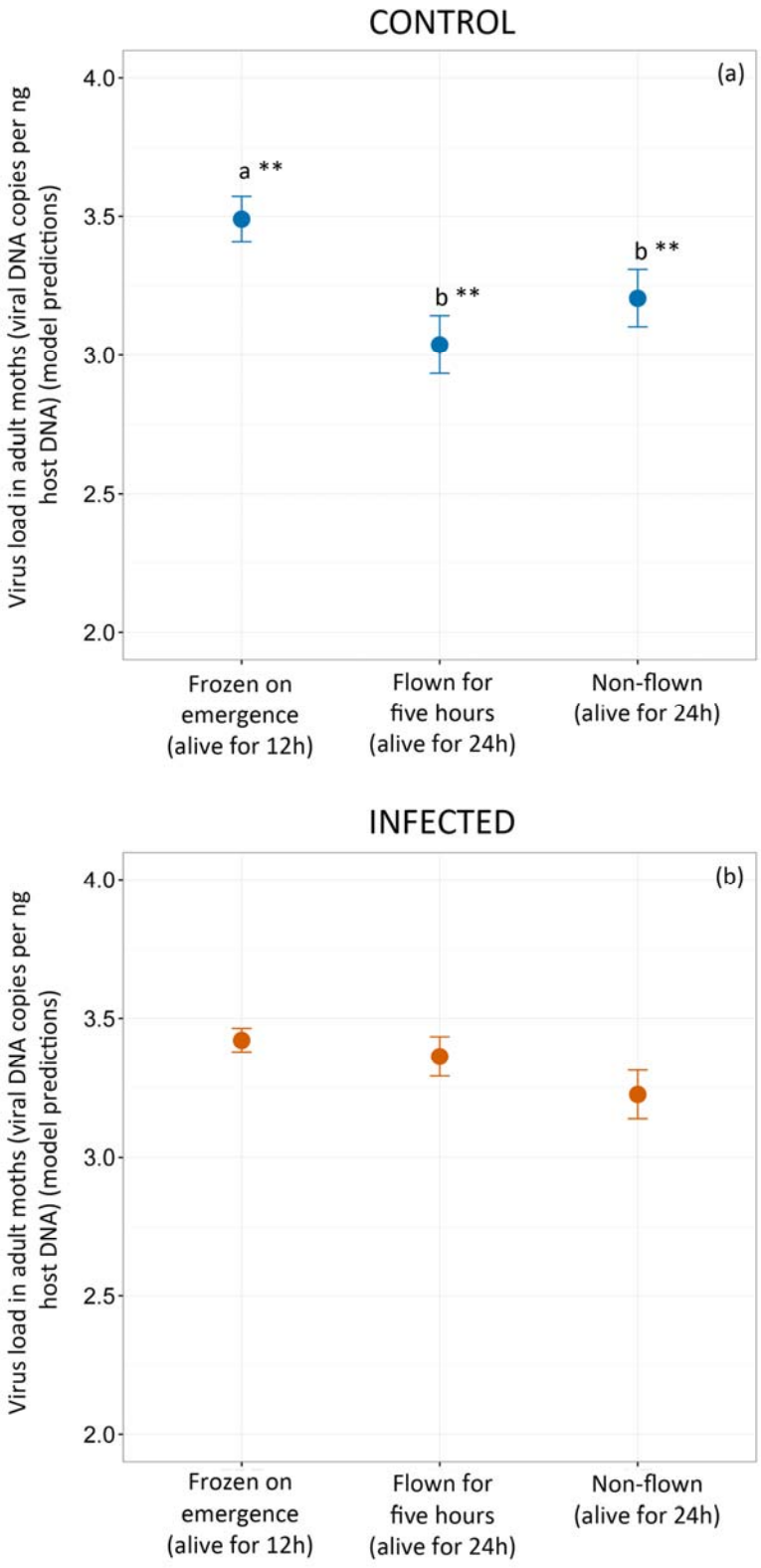

Figure 4.9: The interaction between adult age and virus challenge in the manipulated flight time trial (MFT). In this study there was a significant interaction between flight treatment and whether or not the insects were challenged with the virus as larvae. This interaction was such that adult moths in the control group which were euthanised 24 hours after emergence had significantly lower virus loads than insects killed at 12 hours, regardless of whether or not they were flown for a period of five hours (a). When insects were challenge with the virus (1,000 OBs per µl) this effect was not present and there were no significant differences between treatments. Full statistics are given in the text and parameter estimates for this model are given in Appendix 5. Grey bars show 95% confidence intervals.

Summary

The effect of flight, and its interactions with pathogen challenge and sex, differed in the two experiments. In the 5hFF trial, there was a significant difference between the sexes such that females had lower virus loads than males, in addition to which, flown insects had higher virus loads than non-flown insects (Figure 4.7). In the MFT experiment, neither of these factors was significant and virus load was instead described by an interaction between virus challenge and adult age (Figure 4.8 and Figure 4.9), with insects in the control group showing some level of recovery from infection after a 24-hour period regardless of whether or not they were flown. This level of recovery was not present in individuals which were challenged with the virus as larvae.

4.4.5 Differences in virus load between the two experiments

To understand if the differences in virus load, developmental rates and weight observed the two experiments may be related to differences in virus loads between the two populations, a t-test was used to infer if the underlying level of infection was different across the two populations. This showed that virus load (log10-transformed) across all treatment groups was significantly higher in the manipulated flight time trial ($t_{318.05} = -9.50$, $p < 0.001$, Figure 4.10)

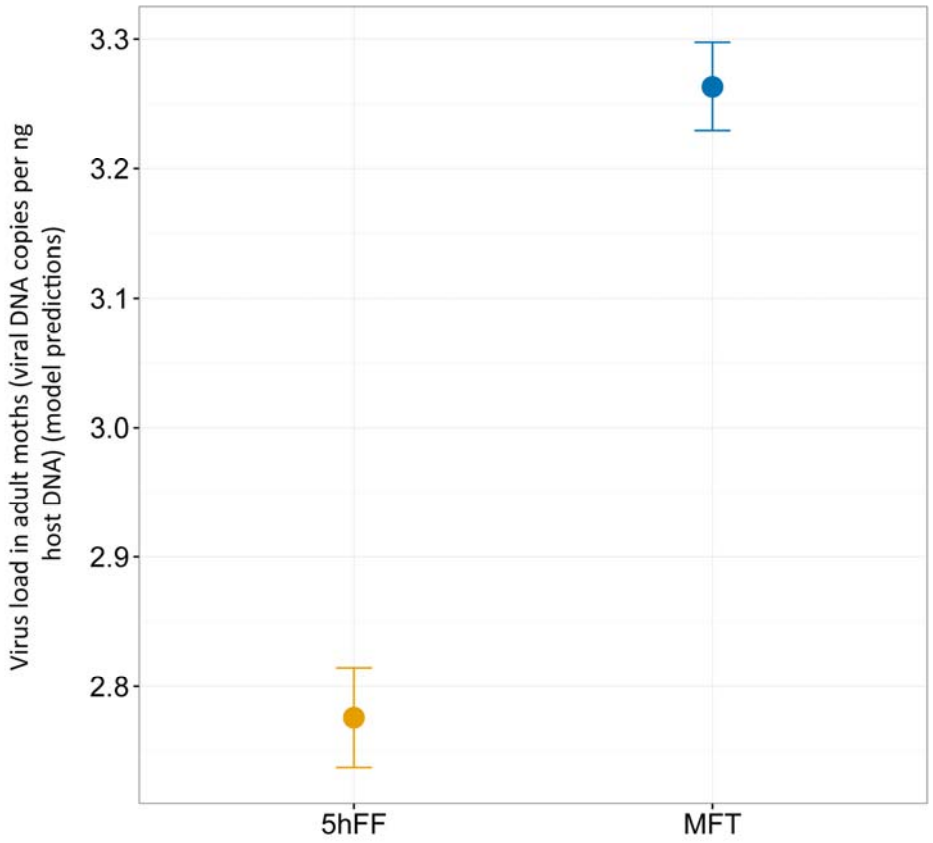

Figure 4.10: Differences in virus load in two manipulated flight trials. When compared using a t-test, virus load was found to be significantly higher overall in the manipulated flight time trial. Full statistics are given in the text. Error bars are 95% confidence intervals.

4.4.6 qPCR REPLICABILITY AND RANGE

In both studies, the range of detection for the qPCR was broadly comparable, 15.38 – 3,811 viral DNA copies per ng of host DNA (1.87 – 3.58 on the log scale) for the five-hour forced flight trial and 49.5 – 15,410 viral DNA copies per ng of host DNA (1.70 – 4.18 on the log scale) for the manipulated flight time trial. In both assays, the limit of detection (defined as the lowest quantity reliably detected in the standard curve) was 10 viral DNA copies per ng host DNA.

4.5 DISCUSSION

The primary aim of these two studies was to assess the cost function between flight effort and disease resistance, a concept which has yet to be tested in the literature (Chapman et al., 2015b).

The first study, a five-hour forced flight trial (5HFF), was designed to quantify the cost function between pathogen exposure and resistance, and assess if this relationship changed after a period of sustained flight effort. The second study was a manipulated flight time (MFT) trial designed to define the cost function between flight effort and resistance, at two different levels of exposure.

(Rolff, 2002)The outcome of these two studies is that physical exertion in migratory systems can lead to changes in pathogen replication rate and host susceptibility (Figure 4.7); a relationship that also varies by sex, with females showing lower virus loads overall, suggesting that migratory tolerance in this system is in accordance with the observations of Bateman's principle (Rolff, 2002) in Chapter 3. Insect fight is among the most energetically demanding biological processes in the animal kingdom (Arrese and Soulages, 2010) and, }, although the shape of the cost function couldn't be determined, this result supports the concept of a trade-off between effort invested in migration and that invested in disease resistance (Chapman et al., 2015b, Altizer et al., 2011)]. The causal mechanism(s) which might drive such changes in migratory resistance as a result of flight effort have not been explicitly investigated here, but there is evidence for mechanisms that might drive this a relationship in the literature. In the desert locust *Schistocerca gregaria*, for example, poor flight capacity in insects infected with the entomopathogenic fungus *Metarhizium anisopliae* var *acridum* was in part due to a reduction in mobile energy reserves (Seyoum et al., 2002). There is also evidence that that immune genes are up-regulated during flight in some migratory insects (C. Jones *et al*., unpublished data), as well as growing evidence that viruses can actively manipulate lipid biosynthesis and, in mammals at least, that anti-viral responses to these lipid-modifying pathways can be modified to limit viral replication (Chukkapalli et al., 2012).

In addition to this effect of physical exertion on resistance, it was also found that insects invest in reducing virus loads in the first 24 hours of the adult lifespan; a resistance mechanism that varies depending on the virus transmission strategy, but not the level of pathogen exposure during the larval phase. Specifically, age post emergence lead to reductions in virus load where exposure to *Sf*MNPV was a result vertical transmission (control populations, Figure 4.9). This relationship is evident regardless of the amount of time invested in flight effort and sex (infected populations, Figure 4.9) but is context dependent. Where insects are exposed to the virus as larvae (horizontal transmission) there are no significant increases in resistance over the adult lifespan. This significant interaction between the age of adult moths and the magnitude of the pathogen challenge (Figure 4.9) is a result that may possibly be driven by different persistence strategies of

vertically- and horizontally-transmitted isolates of the baculovirus species (Cabodevilla et al., 2009) and would support evidence of changes across life history stages as observed by Graham et al. (2015).

However, these results were not consistent across both experiments. Changes in virus load with adult age were not measured in the 5HFF trial, but in the MFT trial, there was no evidence of flight effort affecting virus load in either sex. Exactly what causes these differences is unclear, but there are some indications that they are due to differences in host population between the two experiments. Insects for both experiments were collected from the same location four months apart (June 2015 for 5hFF experiment and October 2015 for the MFT trial). Both were reared in the same facilities under the same conditions, and flown within the first three laboratory generations (section 4.3). Yet many of the development and mortality parameters differed in both experiments. In the MFT trial virus loads were significantly higher on average than those in the 5HFF trial (Figure 4.10) and were accompanied by spontaneous outbreaks of disease in experimental controls (Figure 4.2), which was not observed in the 5HFF trial or Chapter 3.

This gives some preliminary evidence that higher virus loads might alter the interaction between sex and pathogen challenge. In the 5HFF trial, the significant interaction that described the relationship between sex and development time was broadly comparable with and supported observations in Chapter 3. In the MFT trial however, there were significant differences in weight and development times between the sexes, but the effect of pathogen challenge was weak or absent (Figure 4.4 and Figure 4.5). This may suggest that when virus loads are high and there is evidence of spontaneous outbreaks of disease in the baseline population, the reductions in susceptibility observed in females and associated with Bateman's principle (Rolff, 2002) in Chapter 3 and the 5HFF experiment aren't great enough to counter exposure at the larval stage, leading to an equivalence in susceptibility between the two sexes(Rolff, 2002).

This leads to the intriguing idea that the shape of the disease curve between migratory resistance and flight effort is determined primarily by the existing level of *Sf*MNPV infection in the migratory population, with a complex suite of behavioural and physiological adaptions such as sex and flight propensity contributing to the variation in infection levels between populations. Differences in host susceptibility has been shown previously to occur in migratory populations of the fall armyworm (Fuxa, 1987) and certainly, the level of resistance varied in the two experimental populations, and there was evidence that this affected physiological parameters such as development time. In Chapter 3, increases in development time at higher levels of pathogen challenge in females were suggested as a form of migratory escape. This was confirmed by the 5hFF trial, where shorter development times lead to reductions in virus load, and male larvae which took longer to develop had higher virus loads (Figure 4.4). In the MFT trial however, there

was no evidence to support any effect of development time on host susceptibility. This suggests that the initial level of infection is important in determining developmental response and associated resistance/ tolerance mechanisms in source populations. The relationship between weight and virus load in the two trials was more consistent: a negative correlation in both experiments (Figure 4.6). This would suggest that habitat quality could play a role in altering host susceptibility, as observed in the monarch butterfly (de Roode et al., 2008a). In the 5hFF trial this was driven by outliers suggesting that the response is more consistent when virus loads are higher, as in the MFT trial.

(Arrese and Soulages, 2010)What might be driving these differences in the factors that affect resistance is not specifically addressed in this study but one particularly likely candidate appears to be seasonality, which has been shown to affect the dynamics of disease in multiple host-pathogen systems (Altizer et al., 2006) and is likely to be relevant in migratory species. Such variation in susceptibility through the migration season certainly has been demonstrated in both the fall armyworm (Fuxa, 1987) and increases in virus load through the migratory season have been observed recently in the closely related African armyworm *S. exempta,* where late season host populations have been shown to have higher levels of both baculovirus infection and genetic diversity, which is associated with higher levels of spontaneous outbreaks of disease (Chapman et al., 2015b, Myers and Cory, 2016, Graham et al., 2015). This would support the observations of mortality in the MFT control groups (late season migrants collected during the return migration in October), which were not present in the 5hFF population or results obtained in Chapter 3 (early season spring and summer populations). Changes in seasonality might also explain why differences in the sexes were no longer present late in the season. Bateman's principle, which describes how selection on life history traits should favour reproductive success in males and longevity in females, has been hypothesised to lead to changes in disease susceptibility between the sexes. This has been demonstrated in terms of its impact on flight capacity in the fall armyworm (Chapter 3), but in late-season insect migrants which are unable to overwinter or survive at northern latitudes, selection should favour migration in both sexes, perhaps explaining why the sex effect that is so evident in chapter 3 and the 5hFF trial, but is not evident in the MFT trial.

In conclusion, this study demonstrates that flight exertion can affect host susceptibility to infection and pathogen resistance. However, differences in physiological response to pathogen challenge in the host suggest that this relationship is phenotypically plastic in different host populations, possibly driven by changes in seasonality which affect how the host choses to partition its resources. These seasonal effects merit further study.

CHAPTER 5.

SPATIAL AND TEMPORAL INFECTION DYNAMICS IN FIELD POPULATIONS

5.1 ABSTRACT

In migratory host-pathogen systems, the spread of disease is likely to be dependent on the extent to which the host mitigates against pathogen infection. Host susceptibility (the extent to which an individual is sensitive to the effects of infection) will depend on both tolerance (the ability to reduce the fitness impact of a given parasite burden) and resistance (the capacity to reduce or limit infection). This is an important distinction, as resistance may have negative effects on the pathogen, where tolerance does not. Laboratory studies of the fall armyworm *Spodoptera frugiperda* infected with the baculovirus *S. frugiperda* multiple nucleopolyhedrovirus (*Sf*MNPV) have investigated both these cost functions. In terms of the relationship between tolerance and pathogen exposure, females have been shown to exhibit reduced development times and the ability to maintain flight effort at increasing levels of virus exposure, qualities that are likely to enable them to escape habitats with high levels of disease prevalence ('migratory escape'). By contrast, males exhibit reductions in flight capacity which may limit their capacity to undertake long distance movements when infected ('migratory culling') (Chapter 3). Investigations into the effect of flight effort on infection levels support this difference between the sexes, showing that viral loads are lower in females than males but that flight effort results in an increase in susceptibility (i.e. higher virus loads, Chapter 4) in both sexes. These results vary between populations, however, and there is some preliminary evidence that suggests this different response in the two sexes may not occur in late season migrants. To understand how these relationships, translate into infection dynamics in the field, this study uses samples of male insects collected from pheromone traps at 13 field sites across the USA and compares spatial and temporal changes in viral loads over a two-year period in 2012 and 2013. The results demonstrate that this host-pathogen system is relatively resilient to fluctuations in seasonality and host dispersal events. Where variation does occur, viral loads are shown to increase through the migratory season and there is evidence for 'escape' from infection in the northern parts of the species' migratory range. Finally, there is evidence that male infection levels decrease in the late autumn when return migrations would be expected, suggesting that seasonality may alter the level of investment in resistance in this migratory host-pathogen system.

5.2 INTRODUCTION

In migratory systems, the relationship between host susceptibility are likely to be important determinants of infection dynamics over spatial scales (Altizer et al., 2011, Shaw and Binning, 2016, Chapman et al., 2015b) (Chapter 1). As seasonality is a key driver of both migration (Shaw and Couzin, 2013) and disease dynamics (Altizer et al., 2006), we might expect migratory host-pathogen systems to have evolved to respond to these cyclical changes in resource availability. As such, host tolerance (the extent to which an individual can tolerate disease without incurring fitness costs) and resistance (the ability to reduce or limit infection) could be expected to differ in migratory and non-migratory seasons. In turn, differential investment in resistance and tolerance strategies may impact the pathogen load and the level of infectivity in the population.

In the fall armyworm *Spodoptera frugiperda*, laboratory studies have investigated the relationship between susceptibility (in terms of both tolerance and resistance) and flight effort. These studies have shown that females are less susceptible to infection with the baculovirus *S. frugiperda* multiple nucleopolyhedrovirus (SfMNPV) than males (Chapters 3 and 4). This seems to stem from females investing in different life-history strategies, weighing more than males on average and then using these additional resources to increase their development times in response to pathogen challenge and maintain their flight capacity, achieving equivalent flight distances regardless of their level of virus exposure, albeit at the cost of greater weight-loss. Differential investment between the sexes also seems to confer changes in the level of resistance, with lower viral loads observed in females than males (Chapter 4).

These differences between the sexes are described by Bateman's principle, which is the theory that life history strategies should differ between the sexes, as males should invest more resources in mating success while selection should favour female investment in longevity (Bateman, 1948). Evidence consistent with this theory comes from a number of systems (Nunn et al., 2009) and has been linked to investment in immunity, which is often higher in females than in males (Rolff, 2002). In the fall armyworm, however, this relationship is inconsistent and may be linked to infection levels in the population generally or variation in seasonal pressures on infection response. In insects, migration is commonly an inter-generational process and the selective pressures on infection dynamics in a migratory population are likely to differ from those which affect overwintering generations (Chapter 4). Insect flight is amongst the most expensive forms of animal locomotion (Bonte et al., 2012, Reinhold, 1999), and immune challenges have been shown to lead to trade-offs in flight capacity that affect males more than females (Chapter 3) (Dorhout et al., 2011). This strategy, however, will only result in a net benefit to an individual's overall reproductive success if the environmental conditions support habitat continuity that can

support local dispersal both in the adult male and his off-spring. When seasonality is likely to lead to significant reduction in survival, the greatest benefits would be conferred by migration to more suitable breeding grounds. This in turn may cause males to alter their response to infection and invest in dispersal effort regardless of their infection status.

Preliminary evidence suggests that this seasonal response to infection might occur in male fall armyworm moths when infected with *Sf*MNPV, as males sampled from early migratory or overwintering populations show reduced investment in immunity but have a comparable response to females when sampled later in the year (Chapter 4). This is likely to be driven by seasonal climate in the northern parts of the species' migratory range, where freezing conditions in the winter months mean insects cannot survive at latitudes above northern Florida and southern Texas (Luginbill, 1928, Sparks, 1979).

To understand the extent to which infection dynamics may change seasonally in male migratory populations, this study makes use of a large-scale field trial carried out as a part of the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA) Climate Change Program. This field collection used baited pheromone traps to sample populations of male fall armyworm moths for four years from April 2011 to October 2014. Insect samples were collected at 13 different sites across the two known migratory pathways: a western population with overwintering in Texas and migratory events occurring on the western side of the Appalachian Mountains, and an eastern population with overwintering in Florida and migration occurring along the eastern side of the Appalachian Mountains (Nagoshi et al., 2012) (Figure 5.1). Making use of this large insect collection, populations were subsampled and qPCR used to assess how levels of viral infection fluctuated spatially and temporally. Wing, weight and population density measurements were taken from the same dataset and used to test the following hypotheses:

i. Increases in virus load will correlate with higher population densities as observed in *S. exempta* (Chapman et al., 2015b), lower adult weights as observed in Chapter 4 and smaller wing sizes, as observed in the monarch butterfly (Altizer and Oberhauser, 1999)
ii. There will be a quadratic change in virus load, population densities and weight over the annual migratory season. This will be such that low population densities in early season and overwintering populations (where there few opportunities for horizontal transmission and less competition (Rose et al., 2000)) will have higher weights and lower virus loads. As the migratory season progresses, this will lead to higher population densities (Chapman et al., 2015b) with greater opportunities for horizontal transmission and this, in combination with increased dispersal effort, will lead to higher virus loads and lower weights (Chapter 4). As wing length is only a weak predictor of flight effort in this

species (Chapter 4) it will either remain constant or decrease through the migratory season as a cost of viral infection (Altizer and Oberhauser, 1999). This hypothesis was tested at two spatial scales:

a. Across the continental USA
b. In the two known overwintering populations where the species is present all year around (Nueces, Texas and Alachua, Florida).

iii. The temporal changes in (ii) are a result of increased levels of flight effort during the migratory period, such that virus loads will be higher and weights lower (chapter 4) during the migratory season, accompanied by increases in population density (Chapman et al., 2012) when compared with insects in the non-migratory season.

iv. *(Rose et al., 2000, Chapman et al., 2015b, Chapman et al., 2012)*As only the healthiest males will be capable of long-distance flight (Chapter 3), viral loads will be lower in the northern part of the species range as a result of the two non-exclusive mechanisms of 'migratory culling' and 'migratory escape' (Altizer et al., 2011)

5.3 METHODS

5.3.1 INSECT COLLECTIONS

From March 2011 to December 2014, adult fall armyworm moths were collected at 13 different field sites across the USA as a part of the USDA NIFA Climate Change Program, run by the Centre for Medical, Agricultural and Veterinary Entomology's (CMAVE) Insect Behavior and Biocontrol Research Unit at the USDA's Agricultural Research Service (USDA ARS) in Gainesville, Florida. Adult male moths were collected from baited pheromone traps (Universal Moth Traps, International Pheromone Systems, Ltd., UK) containing Hercon Vaportape (active ingredient: 10% dichlorvos or DDVP) daily and shipped to the USDA ARS on ice, where they were stored at -20˚C. Trapping was carried out year-round in Nueces, south Texas and Alachua, north Florida, where *S. frugiperda* is known to overwinter. All other sites, including Orange Country in central Florida, were sampled only in the migratory season (circa mid-April to 31st October). The USDA ARS were also able to provide data on the number of insects caught in the pheromone traps every day, and this was used as a proxy for population trends in each location over time.

Insects were shipped on ice to Rothamsted Research in four batches between January and April 2016. Insects were stored at -20˚C until used in experimental trials.

5.3.2 EXPERIMENTAL DESIGN

The USDA ARS sample collection is extensive, and the data provided was used to design a sub-sampling strategy with the aim of quantifying how virus load fluctuates over time at different spatial scales, and how this varies in the migratory season and for known migratory populations (see section 1.4.1 and Figure 1.3). In addition to virus load, weight and wing length were also measured (section 5.3.4) and the three variables were investigated alongside fluctuations in male population density over time (section 5.3.4).

To account for inter-annual variability, insects were sampled across two of the four available years (2012 and 2013). These were chosen because the years were consecutive and there was the greatest overlap of trap catches in the multiple locations. Of the 13 locations available, samples were only taken from the nine that occurred in both years (Figure 5.1).

Across geographic locations, pheromone trap catches varied widely and it was not possible to sample insects on the same dates in every location. To overcome this, insects within each geographic location were sampled from the first available trap catch in 2012. To avoid sampling from overlapping generations, consecutive sample dates were chosen as the first date that was a minimum of 21 days apart from March to October and at least 28 days apart from November to February, when the temperature drops and development takes longer (Luginbill, 1928). The

maximum number of days between samples was 129. For each sample date within each geographic location, a maximum of 15 moths were selected at random for further analysis. No minimum sample size was imposed.

The final number of samples was 624 insects from nine different locations over two years. Sample availability (number of insects caught) for each location is shown averaged across month in Figure 5.2 for each of the four geographic regions in 2012 and 2013.

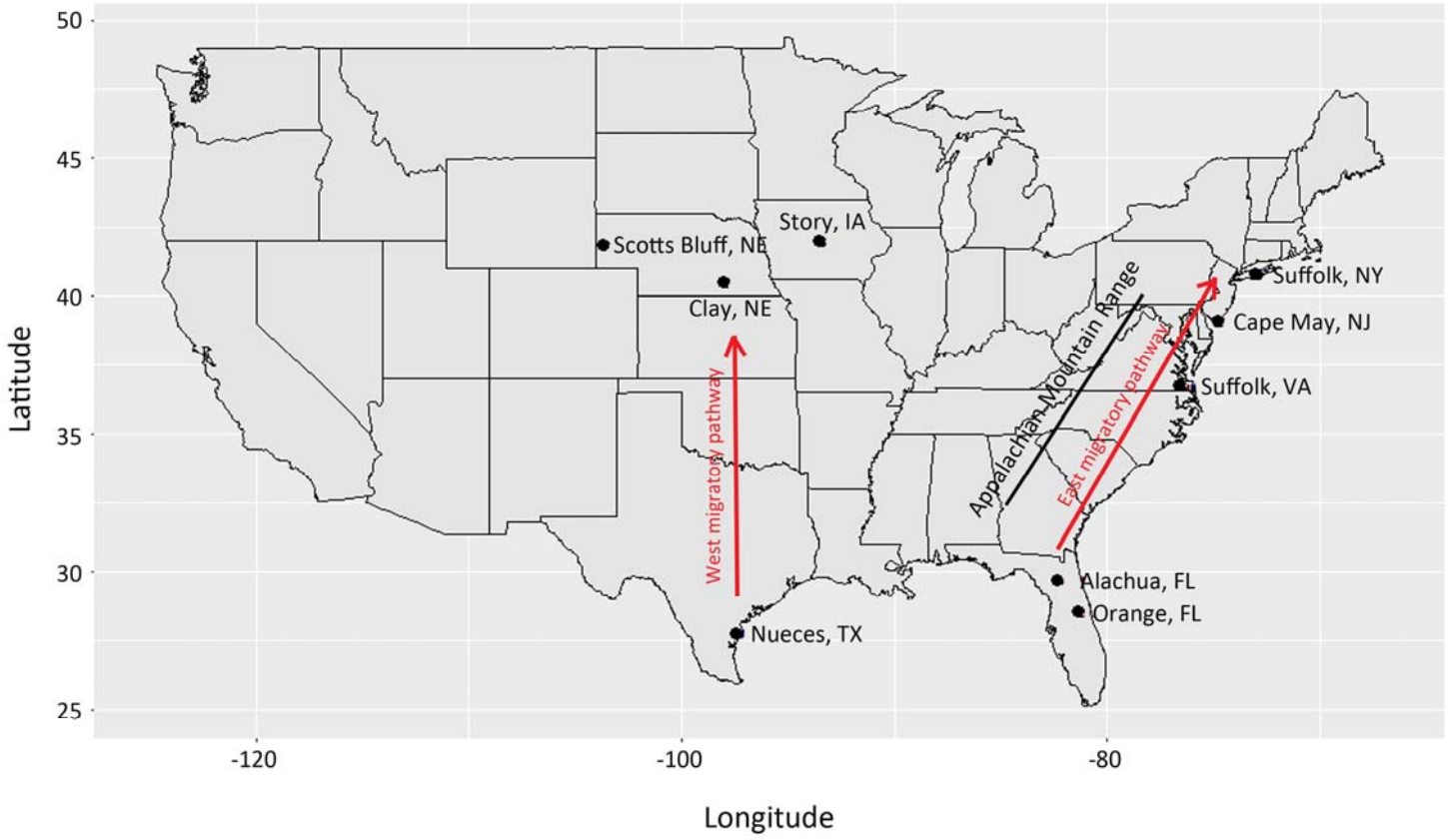

Figure 5.1: Field sampling sites across the USA. Locations of pheromone traps where male insects were collected across the USA in 2012 and 2013. Sites were selected to assess variability along both migratory routes (Nueces, Texas; Clay, Nebraska; Scotts Bluff, Nebraska and Story, Iowa in the West; Orange and Alachua counties in Florida; Suffolk, Virginia; Cape May, New Jersey and Suffolk, New York in the East). Continuous year round sampling was carried out in the over-wintering grounds of Nueces, Texas and Alachua, Florida, where the moths overwinter and are present all year. All other sites were only sampled during the summer migratory season of June through to October.

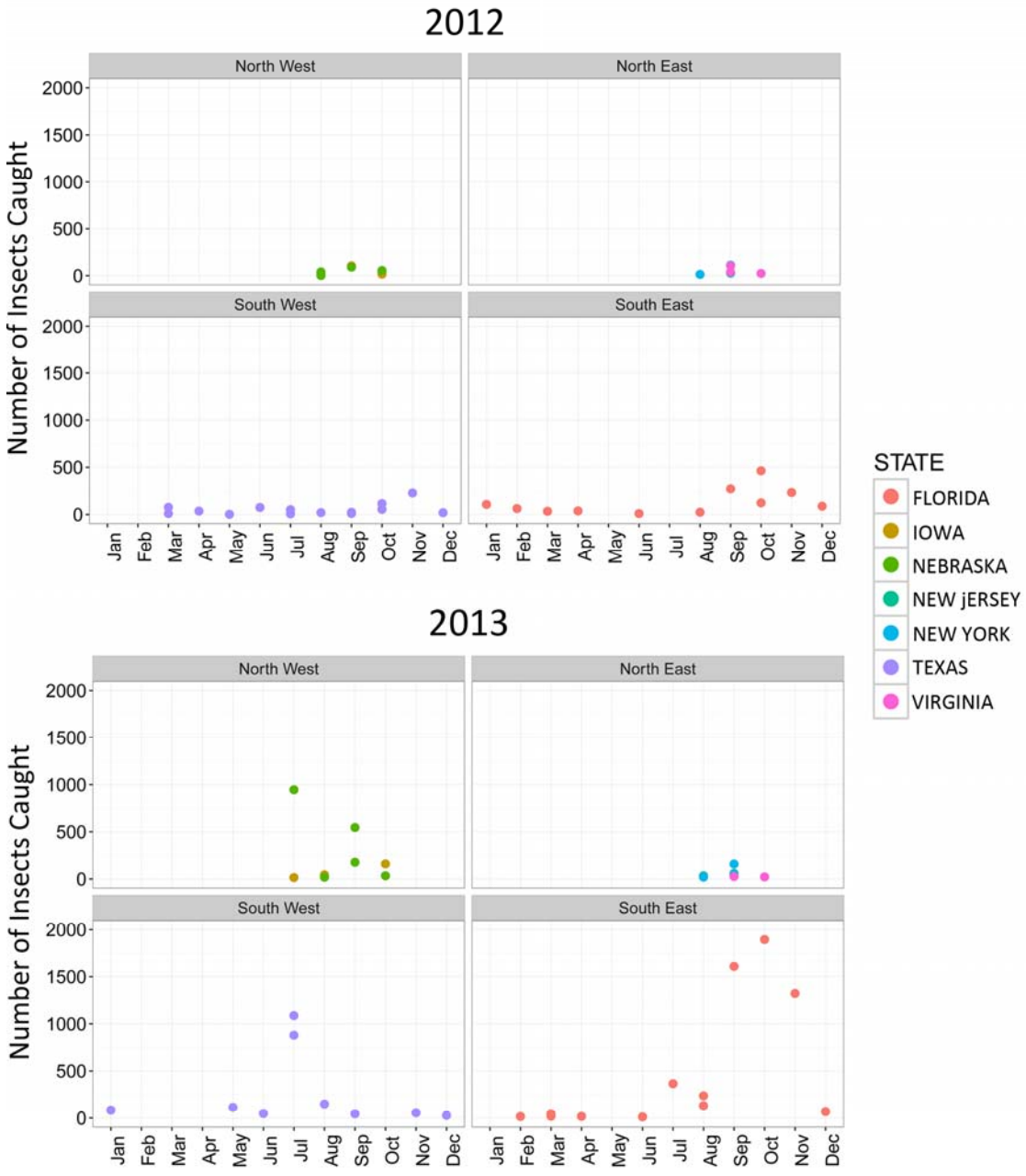

Figure 5.2: Sampling strategy for field data across the four geographic regions in 2012 and 2013. Insects were sampled across four geographic regions (North East, North West, South East, South West, Figure 5.1) in 2012 and 2013. Here the number of insects available (number of insects caught on each of the designated sampling dates) in each geographic location is shown, averaged across month. Different colours represent the contributions of different States.

5.3.3 SAMPLE PROCESSING

Three different measurements were taken from all moths: weight, wing length and virus load. Weight and wing measurements were taken with assistance from Philip Gould, Rothamsted Research.

Moths from the trap catches were surface sterilised by washing them once in 1% sodium hypochlorite solution and then twice in 100% ethanol. Insects were left to dry for approximately 20 minutes and then weighed using a Sartorius R200D balance, accurate to 0.01 mg.

For copy number and wing length measurements, samples were randomly allocated to processing batches of 25 insects. The length of both wings was measured using digital callipers accurate to 0.01 mm and averaged across individuals (see Chapter 3). Where one of the wings was damaged or missing, the measurements were only taken from one wing.

Viral copy number was established using the same protocol described in Chapter 4. Briefly, insects were homogenised using an MG FastPrep-24™ 5G bead beater and DNA extracted using a DNEasy® Blood and Tissue kit (P/N 69506; QIAGEN®, UK), following the insect specific protocol (Appendix 2). DNA purity was assessed using a Nanodrop 2000c spectrophotometer (ThermoFisher Scientific, UK) and quantified using a Qubit® Fluorometer 2.0 (Invitrogen, UK) in conjunction with a Qubit® dsDNA BR Assay Kit (P/N: Q32853, Invitrogen, UK). After extraction, DNA samples were stored at -20˚C.

Copy number was calculated using absolute quantification on a Rotor-Gene Q, using the same protocol described in Chapter 4. Every qPCR run included a maximum of 25 insect samples, a no template control (NTC) and a standard curve run in triplicate. The limit of detection in this trial was 10 viral DNA copies per ng of host DNA and the range of detection fell within 20 – 340,700 viral DNA copies per ng of host DNA (1.30 – 5.32 on the log scale).

As insects were caught using a pheromone trap, it was assumed all samples were male. Where the wings and bodies of the insects were relatively intact, the sex and species was confirmed visually. No females were found, and all insects appeared to be *S. frugiperda*.

5.3.4 STATISTICS

The same linear modelling process described in Chapter 2 was used to investigate relationships in this chapter (detailed in individual sections below). Analysis was carried out in R 3.3.0 “Supposedly Educational” (Pinheiro J et al., 2014) using the nlme (Pinheiro J et al., 2014) and lme4 (Bates et al., 2014) packages.

In combination with pseudo-replication by sample site and the need to account for variation due to processing batches, the unbalanced sampling strategy that was driven by regional variation in trap catch numbers meant the most adequate models for this dataset could not always be fitted. Where relevant, these are discussed in the text.

5.3.4.1 LINEAR RELATIONSHIPS BETWEEN VIRUS LOAD, WEIGHT, WING LENGTH AND POPULATION DENSITY

Hypothesis (i) assumes that increases in virus loads will be accompanied by higher population densities (Chapman et al., 2015b), lower adult weights (Chapter 4) and smaller wing sizes (Altizer and Oberhauser, 1999). To test this, data from all sites and all years was used to investigate the correlations between wing length, weight and number of insects caught in the pheromone traps (a proxy for population density) and virus load.

First, virus load was modelled as a function of the three explanatory terms; weight, wing length and pheromone trap catch. Although this is a large dataset, the complexity of the data (nine sampling sites, each sampled at different time points and considering up to three random terms) did now allow enough degrees of freedom for interactions to be fitted, so each term was modelled individually. Starting models were:

Virus load (log10-transformed) = $\alpha + \beta$ x *Weight* (mg) (log10-transformed)

Virus load (log10-transformed) = $\alpha + \beta$ x *Wing length* (mm)

Virus load (log10-transformed) = $\alpha + \beta$ x *Pheromone trap catch* (log10-transformed)

If the random terms explained a significant amount of variation, the models were corrected for processing batch, pseudo-replication by sampling site and month within year effects.

Relationships between wing length, weight and population density were also assessed using linear models. As there is no obvious causal relationship between population density (trap catch), wing length and weight, the three measurements were modelled as both dependent and independent variables. As this did not change the fundamental relationships, only the results from the starting models below are reported:

Pheromone Trap Catch (log10-transformed) = $\alpha + \beta$ x *Weight* (mg) (log10-transformed)

Pheromone Trap Catch (log10-transformed) = $\alpha + \beta$ x *Wing length* (mm)

Pheromone Trap Catch (mg) (log10-transformed) = $\alpha + \beta$ x *Wing length* (mm)

Again, where pseudo-replication by sampling site and month within year effects explained a significant amount of variation, they were included as random terms. Processing batch was also considered as a random term for wing length.

5.3.4.2 TEMPORAL AND GEOGRAPHIC VARIATION IN VIRUS LOAD, WEIGHT, WING LENGTH AND POPULUATION DENSITY

Per the assumptions of hypothesis (ii) changes in virus load, weight and population density should be low in early season migrants, increasing through the migratory season but reducing again over the overwintering period. As a poor predictor of dispersal in this species, wing length will either remain constant or decrease with increasing virus loads (Altizer and Oberhauser, 1999)

This hypothesis was tested at three spatial scales: (i) across the continental USA using all available data; (ii) in known overwintering populations where the species is present all year around (Nueces, Texas and Alachua, Florida) and (iii) by comparing migratory and non-migratory seasons

Temporal variation in virus load, weight, wing length and population density across continental USA

The nine different sampling locations were categorised by month (January 2012 – December 2013), and virus load, wing length, weight and number of insects caught in the pheromone traps across all populations were individually modelled as a function of time. The starting model for all four variables (pathogen load, wing length, weight and number of insects caught) was:

$$\text{Response} = \alpha + \beta \times \textit{Month}$$

To test for non-linearity, this relationship was modelled using month as both a continuous variable and as a 24-level factor. The two models were compared using the sums of squares of the residuals. Where the two models were significantly different, the trend was assumed to be non-linear. To assess if the relationship with month as a 24-level factor was significantly different from zero, the term was assessed using the standard modelling procedure described in Chapter 2.

Pseudo-replication by sampling location was accounted for as a random intercept in all models. Virus load and wing length models also investigated processing batch as a random term.

It could be argued that these data should be modelled using generalised additive models to investigate non-linearity, but including splines in the random terms resulted in over-parameterisation. For this reason, linear mixed effects models using a factorial analysis were a better fit to the data.

Temporal infection dynamics in habitats with year-round populations (Nueces, Texas and Alachua, Florida)

To understand how dynamics varied over time in a single geographic location, virus load data from Nueces, Texas and Alachua, Florida (the two sites with year-round sampling in both 2012 and 2013) were modelled as a function of time. The data was pooled into four categories, each of which was modelled individually: Nueces, Texas in 2012; Alachua, Florida in 2012; Nueces, Texas in 2013 and Alachua, Florida in 2013. The modelling process for this was the same as that described above, but date was modelled by week of the year rather than by month. To check for non-linearity, models containing week as a continuous variable and week as a factor were compared by assessing differences in the sums of squares of the residuals. The starting model for all four models was:

Virus load (log10) = α + β x *Week Number*

Processing batch was investigated as a random term for all models.

A single model that included both sites in both years, with a three-way interaction between week, location and year would have been an alternative approach but was again over-parametrised so the four separate geographic time points were modelled separately. Where time was a significant factor, weeks were compared using a Tukey test which was implemented using the *glht()* function in the library multcomp for LMEs (Hothorn et al., 2008).

5.3.4.3 DIFFERENCES IN VIRUS LOADS IN KNOWN MIGRATORY POPULATIONS

Comparing virus loads in migratory and non-migratory seasons

To determine if the temporal dynamics observed in section 5.3.4.2 relate to migratory propensity, hypothesis (iii) assumes that the increased levels of flight effort during migration will lead to higher virus loads and decreases in weight (chapter 4), which will be accompanied by increases in population density (Chapman et al., 2012) when compared with insects in the non-migratory season.

This was investigated by pooling all the data into two categories (insects collected during the migratory season and insects not collected during the migratory season) and virus load and weight assessed between the two. A month was defined as 'migratory' if there was evidence of populations in the northern parts of the species' range (August to October in 2012; July to October 2013, Figure 5.2). Sampling date was again categorised by month and the years 2012 and 2013 were modelled separately, using the process described in Chapter 2. This resulted in a two-level factor where months were either migratory or non-migratory, with the starting model:

Virus load (log10-transformed) = α + β x *Migratory Population (Yes/No)*

Random terms tested in the model were sampling site and processing batch. In all models where time (week/month) was found to be a significant explanatory variable for virus load, the same relationship was investigated for wing length, weight and number of insects caught. The modelling process for these was the same as that for virus load.

Geographic and temporal variation in known migratory populations

The fourth and final hypothesis assumed that, as only the healthiest males will be capable of long-distance flight (Chapter 3), viral loads will be lower in the northern part of the species range as a result of the two non-exclusive mechanisms of 'migratory culling' and 'migratory escape' (Altizer et al., 2011)

To test this, data was pooled across the northern and southern parts of the species' migratory range. These were assumed to vary because insects in the north are known migrants, while insects in the south (Texas and Florida) are less well defined and may be long-term residents, migrants from further south or a combination of the two (Nagoshi et al., 2014) (Nagoshi et al., 2007b, Nagoshi et al., 2007c). Again, these data could have been modelled as a three-way interaction between North/South, migratory pathway and year but the need to investigate multiple random terms meant this model was over-parameterised. Instead, the data were subset into four categories: east migratory pathway in 2012, west migratory pathway in 2012, east migratory pathway in 2013 and west migratory pathway in 2013. Within these subsets, only dates where trap catch data was available in both the north and the south were compared (August – October for all datasets except the west migratory pathway in 2013, insects were also caught in the north in July, see Figure 5.2). Data was collapsed over month and modelled as a factor, using the same process described above to investigate the interaction between month of the year and the northern/southern parts of the species' range. The starting model for all four datasets was:

$$\textit{Virus load}\ (\log10\text{-transformed}) = \alpha + \beta1 \times \textit{Month} + \beta2 \times \textit{NorthSouth} + \beta3 \times \textit{Month x NorthSouth}$$

Pseudo-replication over sampling site and processing batch were investigated as random terms.

5.4 RESULTS

5.4.1 RELATIONSHIPS BETWEEN VIRUS LOAD, MOTH WEIGHT, WING LENGTH AND POPULATION DENSITY

Although a number of the four variables measured have been shown to correlate in the laboratory (Chapters 3 and 4), none of the variables in this study were linearly relate to each other ($p > 0.05$, Figure 5.3), suggesting a more complex set of drivers behind the variation in virus load, weight, wing length and population density in field-caught populations.

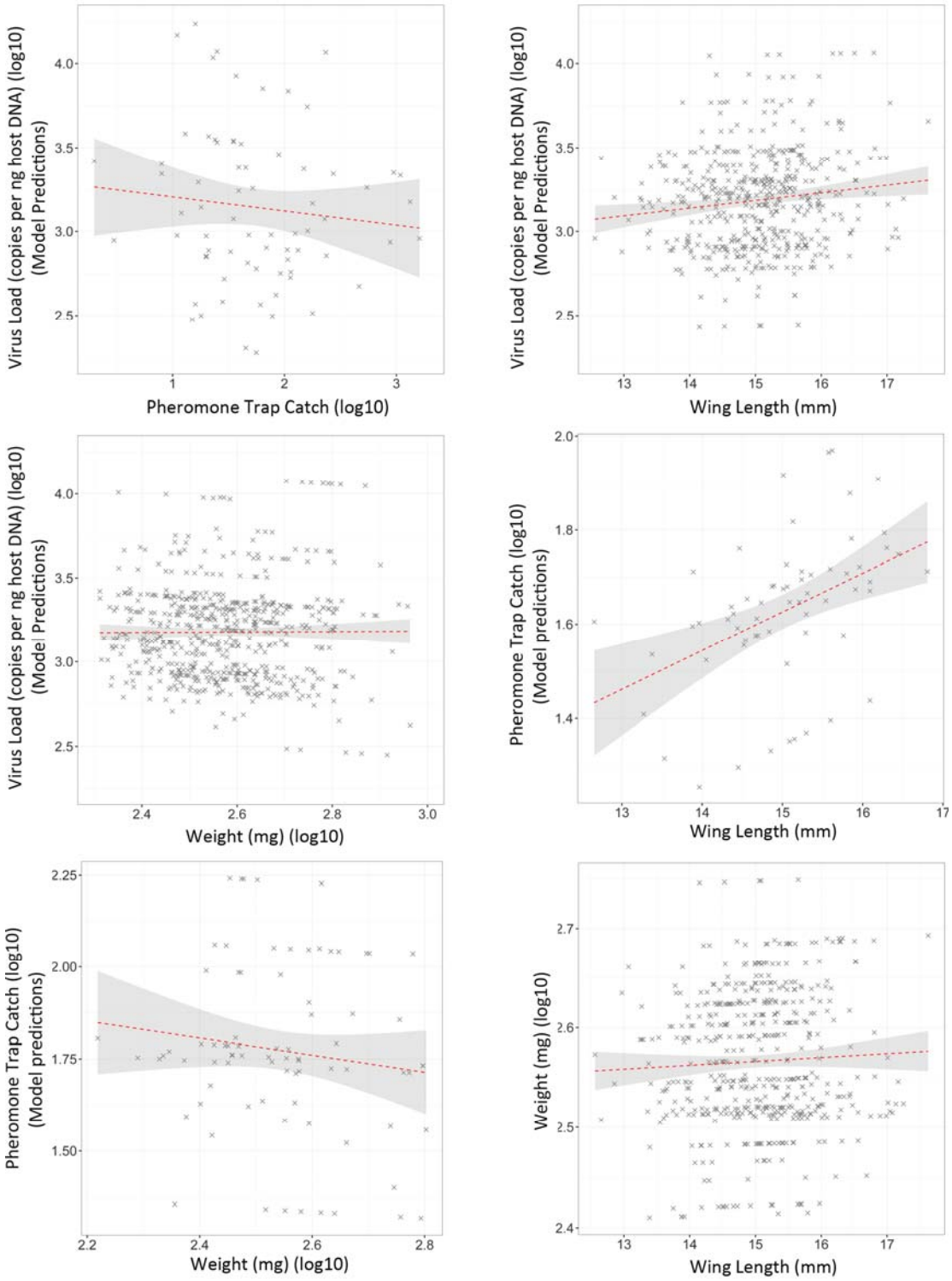

Figure 5.3: The relationships between virus load, weight, wing length and number of insects caught in pheromone traps. Although laboratory studies would suggest causal relationships between the four variables (pathogen load, wing length, weight and number of insects caught), the available field data was highly variable and none of these relationships investigated were significantly different from zero. Parameter estimates are available in Appendix 6. Grey bands show 95% confidence intervals.

5.4.2 TEMPORAL VARIATION IN VIRUS LOAD, MOTH WEIGHT, WING LENGTH AND POPULATION DENSITY

For all variables, with the exception of wing length ($p > 0.05$), calendar month (as a 24-level factor) explained significant variation in each of these traits (*Virus load* LME with processing batch and sample location as random terms: $F = 1.98$, $n = 624$, $p = 0.006$, *Weight* LME sample location as random terms: $F = 8.48$, $n = 624$, $p < 0.001$; *Pheromone trap catch LM*: $F_{23, 55} = 8.48$, $p < 0.001$; Figure 5.4**Error! Reference source not found.**).

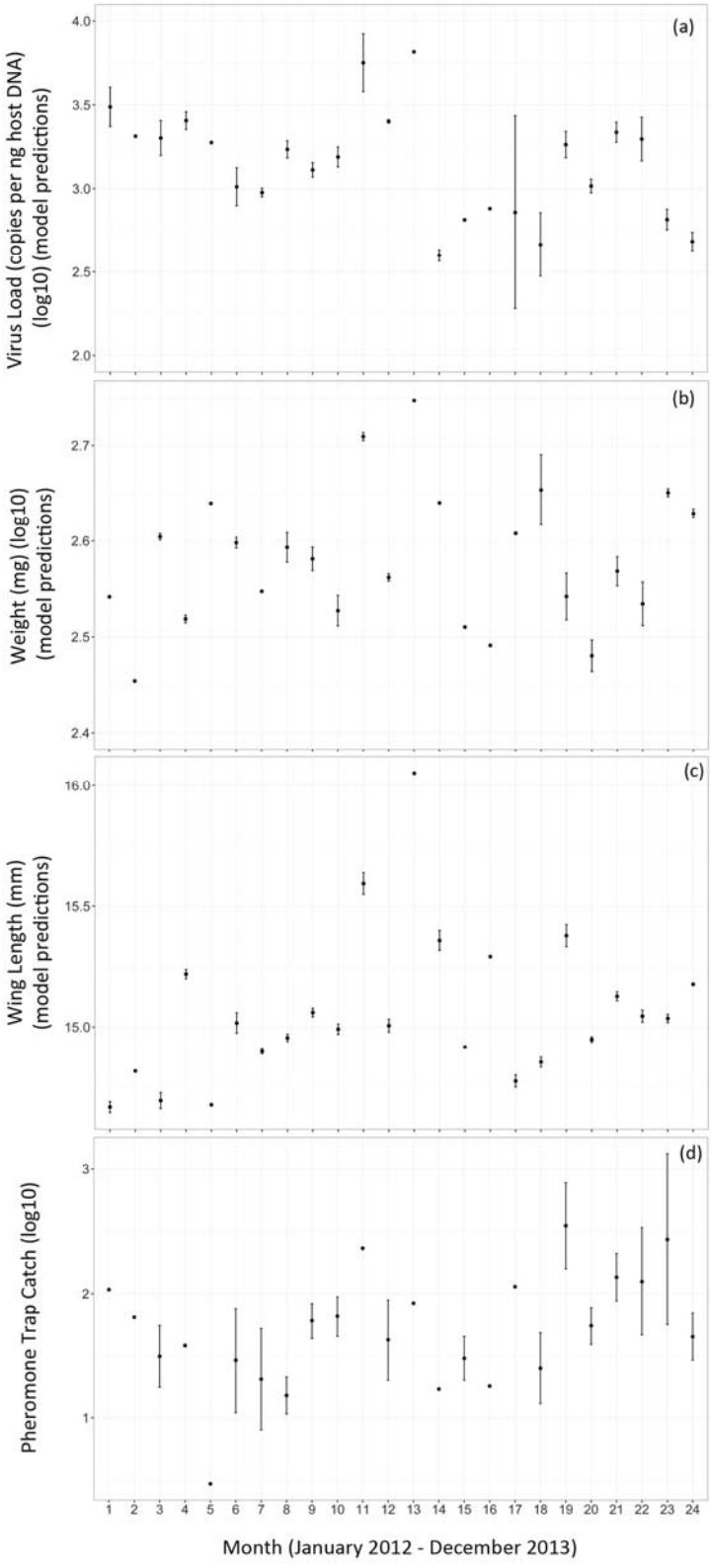

Figure 5.4: Annual variation in virus load, weight, wing length and trap catch data. Model plots of the non-linear trends in the four variables measured over time. When fitted as a 24-level factor there was significant variation by month for virus load, weight and number of insects caught in the pheromone traps (a - c). The relationship for wing length was not significantly different from zero or a standard continuous variable model (d). Error bars show 95% confidence intervals. Statistics are reported in the text and are given in full in Appendix 6.

5.4.3 TEMPORAL INFECTION DYNAMICS IN HABITATS WITH YEAR-ROUND POPULATIONS

When investigating this temporal relationship in more detail, evidence suggests that virus load remains relatively constant over time and in different geographic locations. The factorial week model was not a significant predictor for variation in virus load in Nueces, Texas in 2012, or Alachua, Florida in 2012 and 2013.

However, when included as a factor, week of the year was a significant predictor of virus load (LM: $F_{8, 44} = 3.82$, $p = 0.002$) and moth weight (LM: $F_{8,44} = 12.41$, $p < 0.001$), but not wing length or population density ($p > 0.05$) in Nueces, Texas in 2013. In this instance, significant increases in virus loads were associated with time points when there were significant decreases in moth weight (weeks 27, 30, 33, 39 which encompasses the 01 July – 23rd September 2013; Figure 5.5), with the inverse relationship evident in week 44 (28th October – 03 November 2013, Figure 5.5). However, this relationship was not always consistent. In the second week of the year (early January) increases in viral load occurred when weight increased on average. There are also instances where significant decreases in weight do not reflect changes in virus load (weeks 20 and 24; encompasses 13th May – 16th June 2013; Figure 5.5). Full comparisons are reported in Appendix 6.

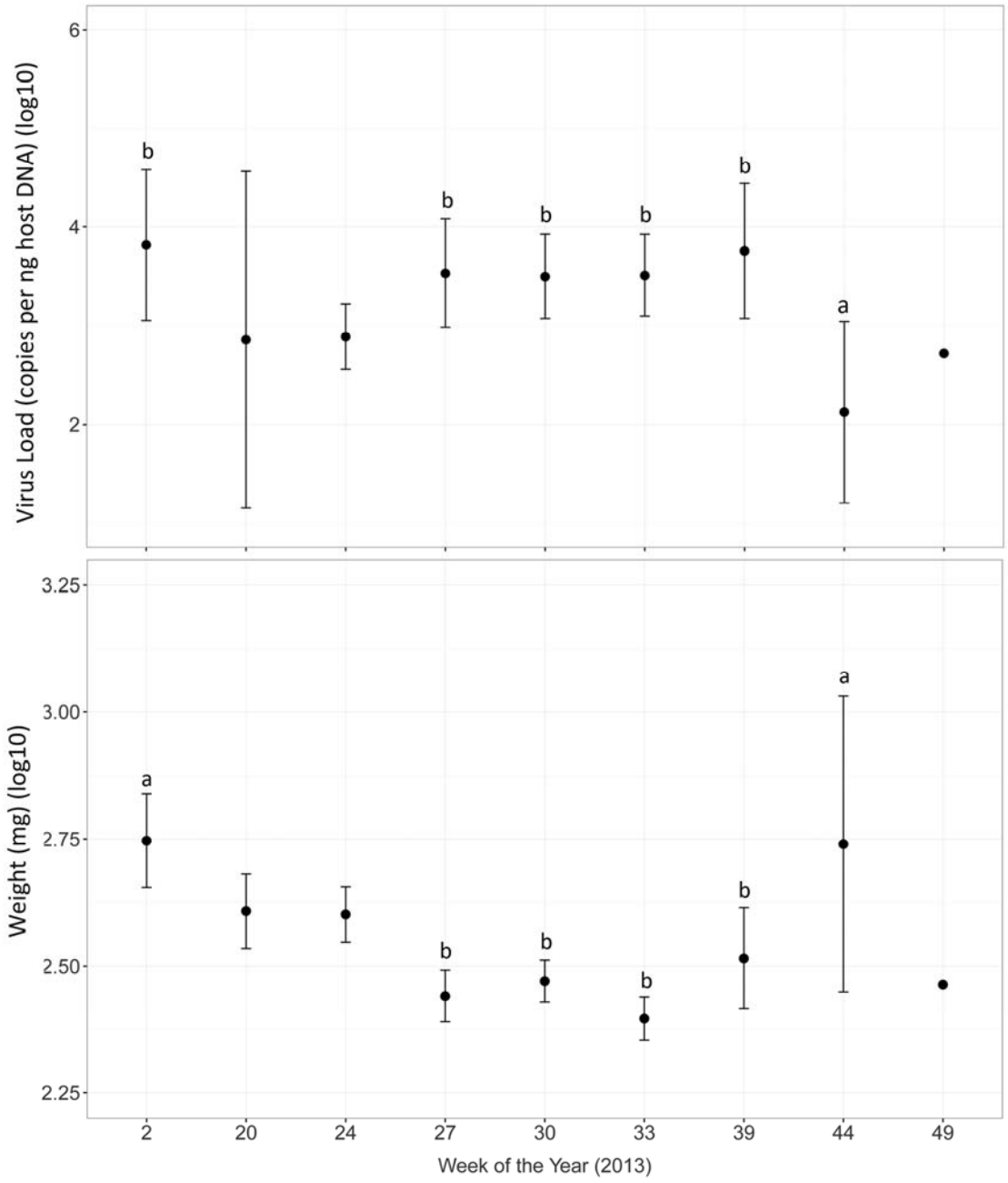

Figure 5.5: Annual variation in viral load and weight of overwintering populations Nueces, Texas in 2013. Mean with 95% confidence intervals for estimates of pathogen load and weight over time in Nueces, Texas, 2013. Results show significant increases in virus load through the migratory season (weeks 27 to 39 which encompasses the 01 July – 23rd September 2013) which decrease significantly in late season migrants (week 44, 28th October – 03 November 2013). These changes in virus load occur in the same time points as significant fluctuations in weight. Model statistics are given in the text and full comparisons reported in Appendix 6.

5.4.4 COMPARING VIRUS LOADS IN MIGRATORY AND NON-MIGRATORY SEASONS

When the months that contained known migratory populations (August to October in 2012; July to October 2013, Figure 5.2) were compared with months that did not contain migratory populations, there was significant variation in both virus load and wing length only in 2013 (2012, $p > 0.05$ for both variables, Figure 5.8a, b).

This relationship was such that virus load was highest in the migratory season (LME with processing batch and sampling location as random terms: $F = 4.58$, $n = 354$, $p = 0.037$, Figure 5.8b), and this was accompanied by a significant decrease in weight on average when compared with the non-migratory season (LME with processing batch and sampling location as random terms: $F = 9.03$, $n = 270$, $p = 0.004$, d).

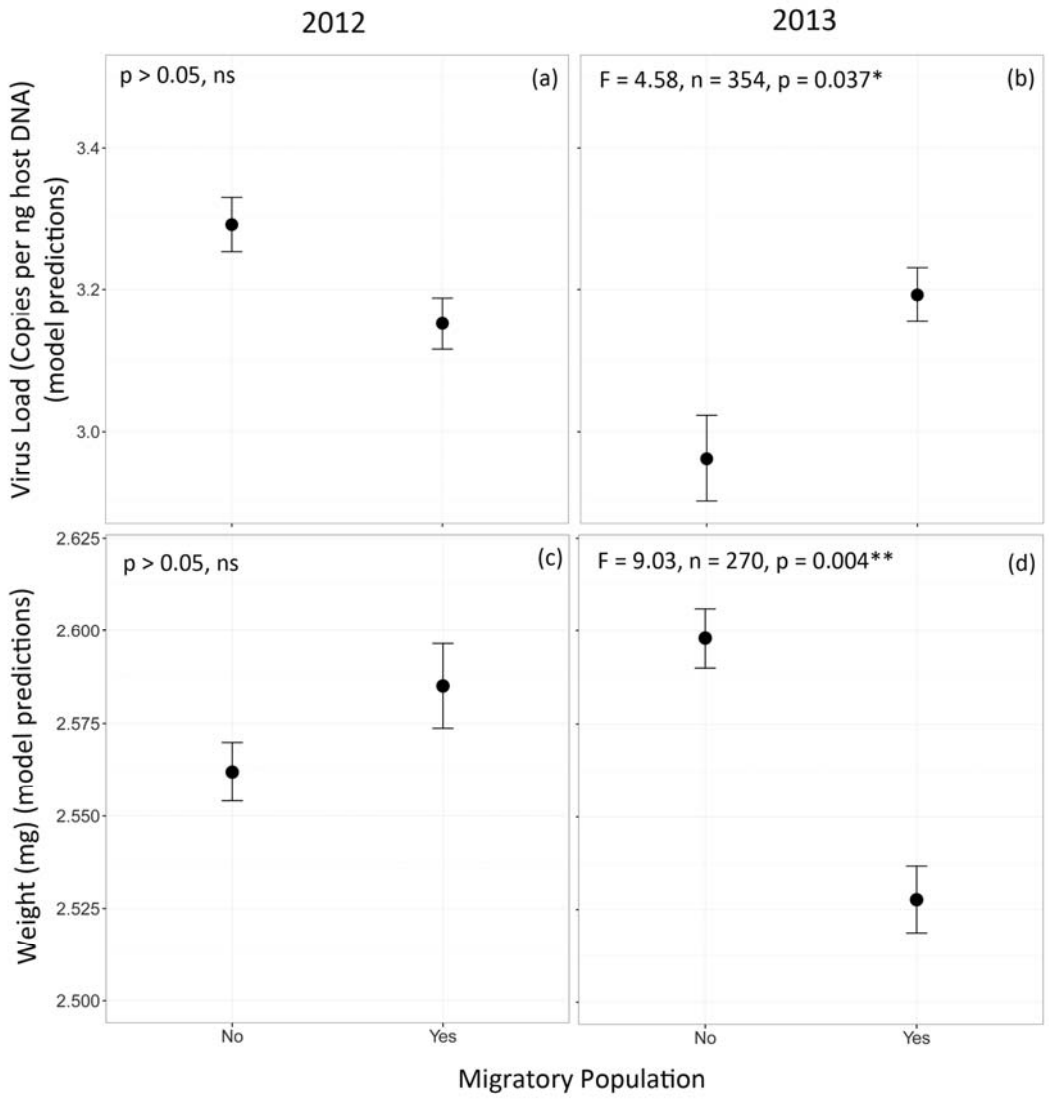

Figure 5.6: Variation in weight and virus load in migratory and non-migratory populations. Results differed for the two years. In 2012, both virus load (a) and weight (c) remained consistent across both the migratory and non-migratory seasons but varied in 2013. This difference in 2013 was such that populations in the migratory season had higher virus loads (b) which co-occurred with significant reductions in weight (d). Full statistics are reported in the text and parameter estimates are given in Appendix 6. Error bars show 95% confidence intervals.

5.4.5 GEOGRAPHIC AND TEMPORAL VARIATION IN KNOWN MIGRATORY POPULATIONS

The differences in virus load between the northern and southern populations differed both by year and location. In 2012, the level of infection was consistent both across different geographic source regions and through time ($p > 0.05$), but in 2013 virus load varied both temporally and spatially, with different dynamics across the two migratory pathways. This also corresponded with changes in moth weight.

Along the western migratory pathway in 2013, virus load (LM: $F_{1, 88} = 20.91$, $p < 0.001$, Figure 5.7 a) and weight (LM: $F_{4, 85} = 5.77$, $p = 0.001$, Figure 5.7 c) varied significantly between the northern and southern populations, such that in the north moths were significantly heavier and had significantly lower virus loads than those in the south in the same time period. This relationship did not vary over the migratory season ($p > 0.05$, Figure 5.8 a, b) and there were no regional or temporal differences in either wing length or the number of insects caught ($p > 0.05$).

These dynamics differed along the eastern migratory pathway for the same period. In this population, the virus load increased significantly in the later part of the season (September and October) (LME with sampling site and processing batch as random terms: $F = 5.25$, $n = 100$, $p = 0.008$, Figure 5.8 b) and these increases were accompanied by increases in weight for the same period (LME with sampling site as a random term: $F = 8.68$, $n = 100$, $p < 0.001$, Figure 5.8 d). This relationship was consistent across both the northern and southern parts of the species' migratory range ($p > 0.05$ for North/ South term, Figure 5.7 a, b) and again there were no regional or temporal differences in wing length or number of insects caught ($p > 0.05$)

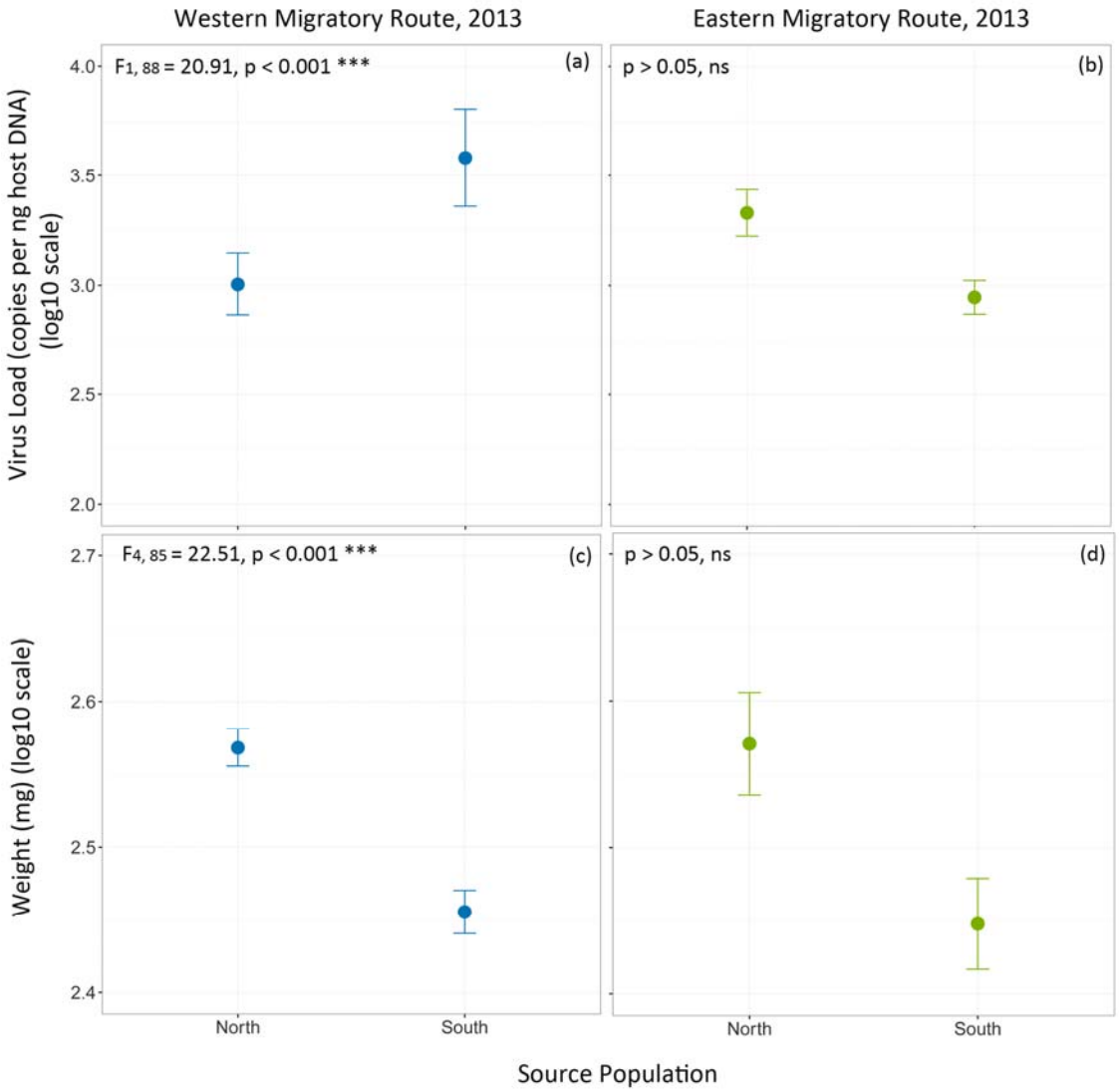

Figure 5.7: Geographic variation in weight and virus load for known migratory populations. Differences in both virus load and weight for male *S. frugiperda* along the eastern and western migratory pathways in 2013. Both virus load (a) and weight (c) varied significantly between the northern and southern populations along the Western migratory pathways such that, in the north, moths were significantly heavier and had significantly lower virus loads than those in the south at the same time period. In the eastern population, both virus load (b) and weight (d) were consistent across the northern and southern part of the species' range. Error bars show the 95% confidence intervals. Statistics are reported in the text, with parameter estimates in Appendix 6.

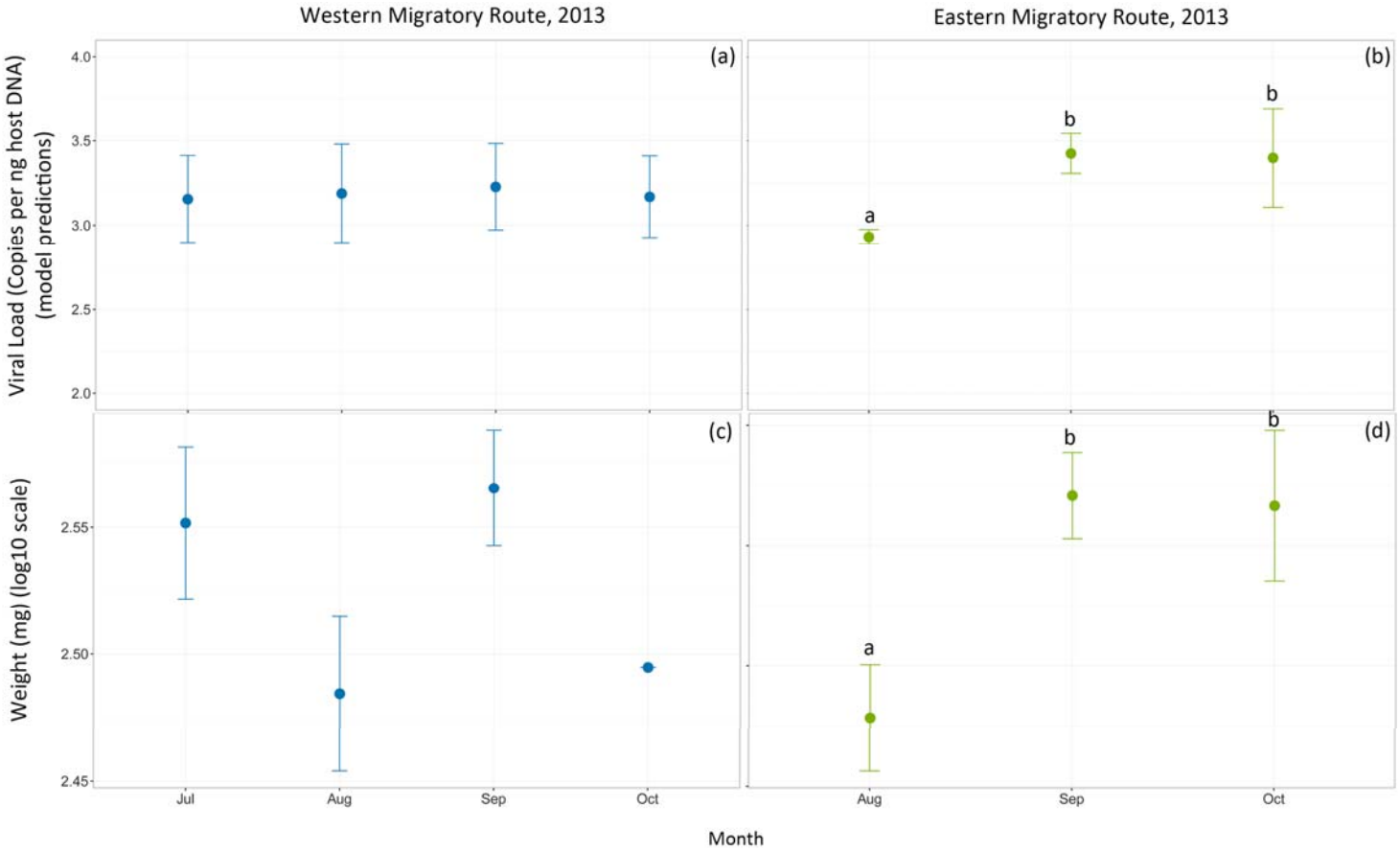

Figure 5.8: Temporal variation in weight and virus load for known migratory populations. Variation in virus load and weight again differed between the two migratory pathways. Along the western migratory route, there were no significant differences in either the level of infection (a) or weight (c) over time. By contrast, in the eastern migratory pathway, significant increases in virus load (b) occurred at the same time as significant increases in weight (d) in the later months of the migratory season. Statistics are given in the text and parameter estimates can be found in Appendix 6. Error bars show 95% confidence intervals.

5.5 DISCUSSION

The aim of this chapter was to quantify the level of seasonal and geographic variation in *Sf*MNPV at 13 different sampling sites across continental USA over a two-year period and assess how variation in the field relates to expectations from the literature and laboratory observations from chapters 3 and 4.

The specific expectations for the way in which migration affects disease dynamics in insects are often based on the monarch butterfly when infected with the protozoan parasite *Ophryocystis elektroscirrha*. Results from this system are very consistent, with several studies supporting the mechanisms of migratory culling and escape, resulting in a decrease in pathogen prevalence over time. As such, this is often used as a template for making inferences into migratory disease dynamics in insect systems. The noctuid-baculovirus migratory disease system is less well defined, and based on what is known this system appears to be far more variable than that of the monarch butterfly. In the African armyworm, the best studied migratory noctuid insect system to date, it is well known that increases in population density through the migratory season result in epizootics in years where there are high levels of rainfall (Rose et al., 2000). (Rose et al., 2000)Early work would suggest that these epizootics are often associated with increases in virus load, but this pattern isn't always consistent and there are also instances where virus load has been found to remain stable or even decrease through the migratory season (Chapman et al., 2015b)(Graham and Wilson, unpublished data). Temporal and seasonal variation in the response to infection would also be supported in the Fall armyworm. In this species exposure to infection has been shown to limit the dispersal capacity of males (Chapter 3), with investment in flight effort resulting in an increase in virus load (Chapter 4). However, not all laboratory studies support these conclusions suggesting that either the underlying level of susceptibility in the host population (Fuxa, 1987) or seasonality (Altizer et al., 2006) may be important drivers in the population level response to infection. In all these studies, the one consistent relationship was hat between weight and the level of infection, with heavier insects having significantly lower levels of infection.

The results from this chapter would support such variability in the overall system, with some underlying support for laboratory observations. In the first year (2012) virus loads remained relatively consistent across the sampling area, but variation in the second year (2013) gives some support to what has been observed in the laboratory. For example, there is evidence male virus load increases in through the migratory season (Figure 5.5, Figure 5.5). This is similar to observations in *S. exempta* (Chapman et al., 2015b)(Graham and Wilson, unpublished data) and might support laboratory observations of dispersal effort leading to an increases in susceptibility

over the migratory period (Chapter 4). The level of infection in the most northern populations was also significantly lower than that in resident populations (Figure 5.7). In conjunction with flight mill studies (chapter 3), this would suggest migratory escape and/or culling might lower infection levels in migratory male fall armyworm populations (Altizer et al., 2011) and is interesting to consider in terms of inter-generational migratory recovery (reduction in infection levels during the migratory period) (Shaw and Binning, 2016). These findings are discussed in detail below.

Geographic and temporal stability in infection levels

Overall virus loads remained very stable both regionally and temporally. This was true in habitats where the species was present all year (Nueces, Texas and Alachua, Florida) as well as in more northern migratory locations, and (Rose et al., 2000)it is interesting to note that infection dynamics in the two separate host populations (Nagoshi et al., 2012) can vary regionally (Figure 5.7 and Figure 5.8). Laboratory studies would suggest that this overall stability in virus levels is likely to result at least partially from compensation mechanisms in the host. For example, shorter development time have been associated with lower virus loads (Chapters 3 and 4) or reducing dispersal behaviour to mitigate against infection (Chapter 3 and 4).

Variation in virus loads during the migratory season

Where variation in virus load does occur, comparisons between populations with and without known migrants would suggest that infection levels are higher during the migratory season (Figure 5.8), and that this may affect entire breeding populations along the length of a migratory route (Figure 5.8) or just areas where there are commonly resident populations (Figure 5.5). Increases in susceptibility through the migratory season is supported by observations in the African armyworm (Chapman et al., 2015b)(Graham and Wilson, unpublished data) with the caveat that these ae highly context dependent and difficult to predict (Rose et al., 2000).

In the majority of cases, this increase in virus load was associated with a decrease in weight (Figure 5.8, Figure 5.8 and Figure 5.8). Reductions in weight and increases in virus load because of flight effort have been observed in the laboratory (Chapter 4) and this raises the interesting possibly that these changes in the fall armyworm my partially be driven by increases in the amount of flight effort required during the migratory season. However, it is difficult to state this with certainty as there is no way of quantifying the level of flight investment in field caught insects (Chapman et al., 2015b).

One notable exception to the relationship between weight and virus load is recorded along the Eastern migratory route in 2013; in this instance increases in virus load were associated with significant *increases* in weight. As mentioned, the relationship between weight and virus load

should be interpreted with caution, but this finding might provide the first preliminary evidence for the migratory recovery as proposed by Shaw and Binning (2016), suggest that where weight increases through the migratory season (potentially due to improved resource availability) males may be able to tolerate higher virus loads regardless of the level of dispersal effort. This in conjunction with laboratory studies (Chapters 3 and 4) suggests that the impact of sublethal infection is strongly related to weight but the relationship is not necessarily causal and may be influenced by environmental factors that are not accounted for here. Wing length would also commonly be expected to increase with weight, but in this study the effect seems to be masked, potentially because this study doesn't take account of energy expenditure over the moths' lifetime.

In addition to this preliminary evidence for migratory recovery, the reduction in disease loads in northern populations (Figure 5.8) suggests the fall armyworm may, in some scenarios, experience migratory 'culling' (infected individuals being unable to undertake migration) and 'escape' (healthy individuals escaping contaminated habitats) as proposed by Altizer et al. (2011). This is supported by flight mill studies in this species have demonstrated that males with the highest levels of pathogen exposure invested less in flight effort (Chapter 3), suggesting the causal mechanism may also in part be behavioural.

Although population densities are known to affect baculovirus infection dynamics in other *Spodoptera* species (Vilaplana et al., 2008), the effect was not evident in this study (Rifkin et al., 2012).

Conclusion

In conclusion, this study demonstrates that sublethal infections of *Sf*MNPV in the fall armyworm are largely similar across space and time, suggesting the relationship between host and pathogen often remains stable, even under fluctuating environmental and dispersal conditions. Where changes do occur though, they may result in increases in pathogen load through the migratory season although more northerly populations may benefit from migratory escape and recovery in some instances and migratory recovery in others. Overall, this data would suggest that the factors driving migratory disease dynamics are highly variable, riven by a complex combination of seasonality, migratory effort and the interplay of biotic and abiotic factors on host susceptibility.

CHAPTER 6.

GENERAL DISCUSSION

The study of the interaction between migration and disease is still an emerging field (Altizer et al., 2011) and the work described in this thesis contributes to the growing body of knowledge in a number of ways. Firstly, Chapter 2 demonstrates the applicability of laboratory-based flight mill systems for the study of flight behaviour and gives insights into the fundamental movement patterns that underlie insect flight in the relative absence of stimuli (Reynolds et al., 2015). The rotational flight mills are then used to quantify the effect of pathogen exposure (Chapter 3) and flight effort (Chapter 4) on disease susceptibility as proposed by Chapman et al. (2015b) (Figure 1.4). These studies provide the first empirical evidence of Bateman's principle affecting the flight response to infection (Chapter 3), with different levels of behavioural and physiological tolerance in the two sexes which in turn is likely to result in varying migratory strategies (Chapter 1). These data also demonstrate that increased flight effort can drive an increase in pathogen load, but show that this response depends on population level infection levels and/or seasonal selective pressures, which might alter the migratory response to infection in a sex-specific manner (Chapter 4). Finally, Chapter 5 looks at infection dynamics in the field and demonstrates that, while virus levels are relatively stable, where infection levels do vary they provide further support for laboratory observations including 'migratory escape' (*sensu* Altizer et al., 2011) in healthy male populations (chapter 5) and 'migratory recovery' (*sensu* Shaw and Binning).

In summary, this thesis describes how host susceptibility interacts with migratory effort to determine infection dynamics. This is discussed in more detail below by first focusing on how the results have contributed to understanding of insect movement behaviour and then focusing on how results from this host-pathogen system give us a fuller understanding of the interaction between migration and disease.

6.1 DEVELOPING THE STUDY OF INSECT MOVEMENT BEHAVIOUR

6.1.1 VALIDATING THE USE OF ROTATIONAL FLIGHT MILLS

Insects are amongst the most rich and diverse of all airborne migrants, their high-altitude windborne movements resulting in the long-range displacement of ~3,200 tonnes per year of biomass above the southern UK alone (Hu et al., 2016). Yet discerning the individual movement patterns of these insects across space and time is challenging, especially under field conditions where technological limitations make it difficult to track movement patterns that can range in scale from a few metres to thousands of kilometres (Chapman et al., 2015b) (Chapter 1). These technological limitations in turn constrain our ability to investigate the effect of the multiple factors which affect this movement. Most notably in relation to this thesis is the impact of disease, yet such considerations are highly relevant to conservation (Altizer et al., 2000), human health (Verhagen et al., 2014) and agriculture (Grzywacz et al., 2008).

To address these questions, many studies instead rely on laboratory techniques to quantify insect flight capacity. Rotational flight mills are amongst the most commonly used (Appendix 1), providing insights into a wide variety of factors from inter-species variation in dispersal capacity (Jones et al., 2016) to the genetic regulation of flight (Jones et al., 2015), as well as a wide range of environmental and physiological factors (Appendix 1). However, this approach has its limitations both in terms of the physical constraints on flight capacity (Riley et al., 1997) and the analysis of the data that are generated (Bruzzone et al., 2009). The results from Chapter 2 address this issue by quantifying the impact that the weight of the flight mill arm has on flight capacity and behaviour, providing empirical evidence that flight mills are likely to result in an underestimate of flight capacity in terms of speed, duration and hence total distance flown. The novel insights in this study are that these decreases in flight propensity are accompanied by an increase in the number of flights, suggesting rotational flight mills can and do capture the underlying drive to fly. In addition, regardless of the limitations imposed by flight mills, descriptions of behaviour are comparable regardless of the weight of the central arm. As number of flights, total distance flown, flight duration and speed are all key measurements of flight capacity in a multitude of rotational flight mill studies (Appendix 1), this additional insight helps give confidence in such studies and contextualise their relevance in terms of other observed field and laboratory behaviours. Extending these studies to other species, however, should be done with caution. Within the insects, there is a wide variety of flight physiology and wing movement patterns (Dickinson et al., 1999) and these are likely to affect the movement capacity and behaviour of different taxa on the flight mills. Work across a broad range of species, however, would suggest that these results are certainly consistent within noctuid moths (Jones et al., 2016) and there is evidence that fundamental movement patterns observed here may be applicable to

dipteran and hymenopteran species (Reynolds et al., 2015). However, similar studies in other taxa would confirm the applicability of these observations more generally and make for useful comparisons, particularly within migratory species.

6.1.2 NOVEL INSIGHTS INTO FLIGHT BEHAVIOUR

In addition to validating the flight mills, the work presented in this thesis gives novel insights into flight behaviour (Chapter 2), describing changes in movement parameters that occurred across all three migratory noctuid species studied – *Spodoptera frugiperda*, *S. exempta* and *Helicoverpa armigera*. In this study, individuals which achieved the greatest total distances did so by undertaking fewer, longer and faster flights. These results were obtained in relatively featureless environments and as such should be interpreted as what might be expected *under conditions of significantly reduced stimuli* (Reynolds et al., 2015, Bruzzone et al., 2009). This point is important because it suggests that the behaviour is effectively innate (Reynolds et al., 2015) and that laboratory manipulations are investigating an underling response mechanism which might be altered in the presence of other external stimuli, as has been shown in other systems (Yamanaka et al., 2001). As such, arguably one of the most interesting of these findings is that all flight parameters varied along a continuum: there was no significant break point at which different movement behaviours might be easily separated (although see the analysis in (Bruzzone et al., 2009). In entomology, the standard definition of migration relates to an individual's physiology and behaviour. Kennedy (1985) described this as a persistent, straightened out movement that results in the temporary inhibition of station-keeping responses, which differs to the approach taken by dispersal ecologists, who tend to focus more on the geographic and population level consequences of animal movement (Clobert et al., 2009). As such, the continuum observed across flight behaviours observed here is important because it means that the results lend support to both definitions. Specifically, the increase in speed, duration and persistence in the insects with the greatest flight potential would support Kennedy's definition of undistracted movement, while the continuity of these behaviours from being stationary to undertaking flights which would have covered more than 50 kilometres in a 12-hour period, imply that long-distance flight under conditions of reduced stimuli is the end of a spectrum of dispersal behaviour upon which selection can act, so driving the evolution of what is observed as migratory behaviour. This is particularly true as one of the other key outcomes of this study (Chapter 2) was that the same continuum of flight behaviours, and increases in flight propensity with increasing distance, was observed both within individuals as well as across populations. Results such as these, properly interpreted, could begin to unite the definition of migration across fields, combining the physiological and behavioural traits at an individual level with ecological and population level consequences (Chapter 1).

6.1.3 Describing changes to flight behaviour in response to pathogen challenge

In recent years, many studies have sought to describe the extent to which disease exposure and susceptibility affects tolerance in migratory systems (Chapter 1). Most these studies are field-based and usually focus on mammals or birds, with the response to infection measured in terms of immune response or migratory distances covered for varying levels of pathogen challenge. Fewer studies have attempted to quantify the direct effects of infection on changes in movement behaviour, specifically addressing the question of if infected hosts alter their movement behaviour to mitigate against the effects of infection. Previous studies using rotational flight mills have tended to focus on the effect of pathogen infection on flight capacity (the total amount of effort invested in flight, usually measured in terms of total distance travelled) (Satterfield et al., 2015, Dorhout et al., 2011), and the insights from Chapter 2 meant that this question could be addressed for the first time in relation to flight behaviour (a multitude of different behaviours which are under the control of an individual such as duration, speed and number of movement bouts which determine overall flight capacity). The results showed a sexual dimorphism in response, with female flight behaviour apparently unaffected by the magnitude of virus challenge while males showed a significant reduction in flight capacity. As the levels of infection increased, males appeared to change their flight strategy completely. Long, uninterrupted flights in the control groups and at low levels of exposure fit the Kennedy (1985) definition of persistent, straightened out movement. At intermediate and higher levels of infection this no longer holds true, with insects investing less effort in flight as virus exposure increases. The shape of this relationship is interesting, as it suggests that males in the intermediate category undertake shorter flights but more of them, a description that might fit better with ranging or foraging behaviour (Dingle, 2014a). At the highest level of pathogen exposure, for whom the pathogen cost is highest, males fly significantly reduced distances and undertake very few flights, suggesting that virus challenge at this level not only affects migratory capacity but even the propensity to disperse. Such a detailed description is novel and begins to demonstrate how the distribution of different movement paradigms across a population might vary because of pathogen exposure, which could have population level consequences.

6.2 Host susceptibility in a migratory system

6.2.1 Bateman's principle and disease tolerance in migratory systems

In addition to the observed differences in flight behaviour following larval challenge with a viral pathogen, there were also marked differences between the sexes in terms of larval/pupal development time and adult weight-loss during flight (Chapters 3 and 4). In Chapter 3 and the five hour forced flight trial in Chapter 4, females were shown to develop faster than males in response to increasing levels of virus challenge (Figure 3.7 and Figure 4.5). This, in conjunction

with the different flight responses, suggests that the physiological response to infection differs between males and females. This is a commonly observed phenomenon (Rolff, 2002, Nunn et al., 2009, Moore and Wilson, 2002), which has been argued to be driven by Bateman's principle (Bateman, 1948). This dictates that selective pressures should favour mating success in males and longevity in females, resulting in different life history strategies in the two sexes. There is certainly evidence for this difference in *S. frugiperda*, with females developing faster, weighing more and having longer wing spans than males on average. Differences in the flight capacity of the two sexes have also been observed in closely related *Spodoptera* species, where a trade-off between flight and reproduction has been well documented (Gunn and Gatehouse, 1993, Gunn et al., 1989). This would suggest different levels of investment in immune response by the two sexes, particularly as females in both experiments lost increasing amounts of weight in response to virus challenge (Figure 3.8 and Figure 4.5), which is supported by a significantly lower level of viral copy number in females than males (Figure 4.7).

Conceptualising this relationship within the context of movement ecology, reductions in flight behaviour at higher levels of infection would suggest that only the healthies males undertake long distance flight (migratory culling, *sensu* Altizer et al., 2011). As these are the insects exposed to the lowest levels of virus exposure, this is likely to result in migratory escape (*sensu* Altizer et al., 2011) and there is some evidence for this in the field in males (Figure 5.7). In females, the relationship between virus challenge and flight behaviour suggests a more complex interplay with the life-history strategy of infected individuals. In these experiments, females showed an increase in development time in response to increasing virus exposure. This fits not only with the concept of migratory escape but, in combination with the flight mill data showing that females invest in flight at the cost of increasing weight-loss, also appears to support the notion that infection acts as a driver of migration in this species. Evidence for such drivers is rare as it requires experimental manipulation of exposure prior to monitoring dispersal capacity in the field, which is limited largely by the technological limitations discussed above and in Chapter 1. Drawing conclusions from a single study in a system where responses have been shown to be variable should therefore be done with caution, but this is a useful addition to the literature.

6.2.2 Flight effort and the factors affecting pathogen loads

The primary point to notice in all the experiments described in this thesis is that there was no direct correlation between the level of virus challenge (as larvae) and virus load (as adult moth). This is somewhat contradictory to expectations, as other authors have shown a clear link between infection in the larval stage and overall sublethal prevalence in the adult population (Cabodevilla et al., 2011b). However, the fact that multiple experiments generated the same consistent result would suggest that the finding is robust. Nonetheless, there may be other fitness effects

associated with large virus challenges, such as increased weight-loss during flight and reductions in flight capacity. Potentially more relevant given the level of geographic and temporal stability in virus load in field populations (Chapter 5), is that the evidence presented here would suggest that the host population invests in resistance mechanisms (physiological and behavioural) that result in an overall level of stability in sublethal infection regardless of the level of exposure.

In this thesis, there is evidence from both field and laboratory trials that, while such stability of the host-pathogen interaction does occur, there are factors which can drive significant variation. These include differences between the sexes, the amount of flight effort, development time and weight, as well as virus transmission strategy. Of primary interest given the hypotheses tested in this thesis is the effect of flight effort. There is evidence from the flight mill experiments that the effort invested in flight over a period of five hours can lead to a significant increase in the level of viral infection, regardless of sex (Figure 5.7). This would seem to transfer to the field, where populations in known migratory seasons, which presumably invest more effort in dispersal on average, have higher virus loads (Figure 5.5 and Figure 5.8). In males, this would seem to be accompanied by an overall decrease in weight on average during the migratory season (Figure 5.5) which would also be supported by observations of flight effort in the laboratory. Although lower on average in the migratory season, the weight of males did increase through the summer in some locations (Figure 5.7) and this would seem to suggest that improved resource availability might mitigate against some of the costs observed in the laboratory. Certainly, heavier larvae in the laboratory were better able to process infection and maintain lower levels of infection, both in these studies and in others (Cabodevilla et al., 2011b). It is also interesting to note that this increase in weight in the field is not always accompanied by a decrease in the level of infection (Figure 5.8), suggesting that through the migratory season males do not necessarily use this additional resource for virus resistance, and where reductions in viral load do occur these are more likely to be as a result of 'migratory escape' (Figure 5.7). This, at least, would be in line with Bateman's principle and observations of behaviour on the flight mills as discussed above.

The observations relating to Bateman's principle, however, seem to break down in late season migratory populations. This was observed in two laboratory experiments, where replication of the same five-hour forced flight treatment in comparison to non-flown controls resulted in significant *increases* in virus load in early-season migrants (Figure 4.10), but not in late-season migrants which had significantly higher levels of sublethal infection prior to viral challenge (Figure 4.9). In this latter population, virus load was determined by if insects were exposed to the virus as third instar larvae. Specifically, control insects (harbouring virus that was vertically-transmitted from their parents) showed a capacity to reduce viral infection over time, an effect that was not present in insects which were orally challenged. While such experiments cannot rule out

differences in viral strain (that which was naturally present in the population versus the Nicaraguan strain used to infect the larvae), reductions in virulence have been observed in isolates adapted for vertical transmission (Cabodevilla et al., 2009). This reduction in virus load over time in the manipulated flight trial, which resulted in lower virus loads overall in males and females, also supports field observations which saw virus loads reduce significantly in late-season male migrants compared to early-season non-migrants. Seasonality is a common driver of disease dynamics (Altizer et al., 2006). and in the fall armyworm, which cannot withstand freezing conditions in the northern parts of its range (Luginbill, 1928), changes in physiology that prioritise migration later in the season are likely to result in the greatest levels of reproductive success. Whether such changes in physiology that prioritise migration are linked to infection mitigation is not clear, but comparable development times and disease response by females in late season populations studied in the laboratory seem to suggest this is a possibility. This is particularly true as male insects might invest in immune responses which prolong longevity and reduce infection from multiple different pathogens (Schmid Hempel, 2011), not only the baculovirus studied here. As such, the reductions in virus load may be in response to a wider level change in immune function and could, for example, be driven by other cues that affect migratory response such as those affecting biological clocks (Dingle, 2014a).

The final point of worthwhile comparison for this work is with what is known of baculovirus disease dynamics in other noctuid species. Of the many species known to be susceptible to infection by a nucleopolyhedrovirus, the infection dynamics have been particularly well studied in the African armyworm, *Spodoptera exempta*, and the western tent caterpillar, *Malacosoma californicum pluviale*. Even in these two systems, there is evidence of how species-specific baculovirus infection dynamics can be. In the western tent caterpillar, the population dynamics are cyclical and driven by spontaneous outbreaks of disease (Myers and Cory, 2016). The spatial structure of the virus is hierarchical: more similar between families, than between populations and across different islands. In the African armyworm, however, infection dynamics are predominantly seasonal, driven by windborne movements of moths in the rainy season, resulting in large scale levels of convergence and population outbreaks in which large-scale epidemics are observed (Rose et al., 2000). In these populations, the levels *S. exempta* NPV (*Spex*NPV) increase through the season alongside increasing baculovirus genetic diversity (Chapman et al., 2015b). In the fall armyworm, outbreaks of disease do occur but they are relatively rare and appear to becoming more so in recent years (Johnson, 1987). As the data here covers only two years, it is not possible to describe how levels of sublethal infection might contribute to the long-term infection dynamics in North America, but the results would support a model where long-term pathogen load is relatively stable and only occasional susceptible to fluctuation. This is interesting in terms of how migratory-disease relationships are defined (Chapter 1). While most studies focus

on one or two mechanisms that drive the geographic presence of disease in migratory populations, the work presented here would suggest that host-pathogen populations can be adapted to the migratory nature of the host. In such systems, where sublethal infection is present in a large proportion of the population and most individuals are carriers, migration does result in migratory spread but this is unlikely to have much consequence. Instead, focusing on the conditions that drive fluctuations in these systems is likely to be more important and the work in this system would suggest that the migratory mechanisms behind these fluctuations (culling, escape, recovery, etc.) are likely to be multiple, with different mechanisms operating at different spatial scales. In this respect, it becomes even more important to understand how cost functions vary in response to physiological and environmental conditions. Being able to determine the impact of these costs at different spatial and temporal scales is likely to be key for managing and mitigating against the migratory spread of disease in ecologically and economically important species.

6.2.3 Implications for pest management

Current legislation is pushing agriculture away from its dependency on agrochemicals and towards ever greater levels of integrated pest management (Ehlers, 2011), and in Lepidoptera-baculovirus systems, the outbreak of large-scale epizootics in some species has led to the use of insect viruses as biological pesticides (Mishra, 1998). However, the complexity of these results demonstrates some of the challenges faced when using natural systems for managing pest populations. Understanding baculoviruses and their implications for population dynamics requires in-depth knowledge of a complex ecological processes (Myers and Cory, 2016, Cory, 2015, Cory and Myers, 2003) and what causes spontaneous outbreaks of disease in natural populations is still largely unknown. Movement and the underlying level of infection are assumed to influence this process (Altizer et al., 2011, Chapman et al., 2015b) but the relative importance of these different factors remains largely unknown.

The work in this thesis would suggest that the relationship between migratory effort and pathogen load is highly context dependant. Sex, the underlying level of infection, migratory effort and weight were all factors in affecting the outcome between host and pathogen (Chapters 3 and 4), and Chapter 5 demonstrates how variable the outcome of these interactions is at different spatial and temporal scales. As the time to kill is much lower in baculoviruses than conventional pesticides (Mishra, 1998), they are unlikely to be able to limit the damage to crops in the same way that chemical pesticides can. An alternative approach is to use baculoviruses to keep pest populations low so that they remain below the economic damage threshold. In migratory species, this is complicated as it requires managing pest populations in one geographic location to reduce the impact in other locations several hundred kilometres away; and where those locations are

largely unknown and dictated predominantly by the weather (Johnson, 1995). With the implementation of a large-scale monitoring network that takes a combined approach of tracking insects with vertical looking and weather radar, using remote sensing to identify suitable habitat and large scale population sampling this approach is labour intensive but possible. However, if baculoviruses are to be used effectively, there is a much greater need of coordinated ecological and behavioural research to identify how the response to infection changes under different biotic and abiotic stressors. This thesis provides an initial starting point for that work, demonstrating how carefully replicated laboratory studies can be used to detect differential responses across populations and validating flight mills as a means of investigating the effect of migratory effort. However, these studies only serve to highlight how much more is coordinated and collaborative research is required before baculoviruses can be used to limit pest populations in this migratory insect system.

6.3 FURTHER WORK:

There are several obvious avenues for further work that would enhance the observations in this thesis. First and foremost is a more rigorous test of the impact of seasonality of virus prevalence. Designing large-scale field studies to monitor and assess these fluctuations are complex but where these studies could be directly linked to other drivers such as weather and climate, habitat availability and population demographics, monitoring infection loads in this way is likely to generate crucial insights into how pest populations might be manipulated with biological pesticides such as *Sf*MNPV. The molecular approach required to quantify pathogen load, however, makes such trials both time consuming and expensive. Using laboratory studies to estimate parameters for ecological models that can be tested against field populations may be a more strategic approach. The work here highlights how important it is to incorporate, develop and test relevant ecological theory to support the building and maintenance of such models.

While the work in this thesis describes resistance and tolerance strategies, it does not cover the mechanisms which might drive those strategies. Understanding immunological measures in different populations and relating them to infection loads under experimentally manipulated circumstances is crucial and would help illuminate cause and effect. By taking into account virus transcriptomics and comparing the results of upregulation in both host and pathogen to what is known about gene expression as part of the migratory syndrome (Jones et al., 2015) it is also possible to understand how these interactions between migration and infection unfold at a molecular level. This has not been attempted, but is possible by combining flight mill studies with pathogen bioassays, where experimental manipulations can be used to investigate the impact of a variety of influencing factors such as sex and flight effort (see Appendix 1).

The costs of infection could also be considered further. Two key factors which are likely to impact the outcome of infection in migratory systems are the utilisation of energy reserves and, at a population level, investment in reproduction. Pathogen exposure for example has been shown to alter the ratio between protein and carbohydrate intake of infected insects (Povey et al., 2009, Povey et al., 2014) and a logical next step for such work would be to understand how this affects an individual's capacity to engage in energetically costly behaviours such as flight. Although reproduction was investigated as a part of this PhD, the need to combine survivorship data with pathogen load measurements taken directly after flight made it difficult to quantify reproductive effort in a meaningful way. Large, inter-generational studies would address this problem and may prove key measurements for understanding how even sublethal infection with *Sf*MNPV may be used to limit population densities in migratory and highly dispersive populations.

Finally, the only aspect of the pathogen's phenotype which was monitored in relation to the impact of flight effort in this thesis was virus load. Investigating other traits is likely to lead to a fuller picture of the interaction between host and pathogen population. One question which is relevant given the literature is how different genetic isolates might vary in their response to migratory effort. For example, (Cabodevilla et al., 2011a) has shown that vertically- and horizontally-transmitted isolates of *S. exigua* MNPV differ in their pathogenicity and virulence. In migratory hosts, long-distance movement could select for different genetic assemblages with different selective pressures affecting intergenerational population level factors such as reproduction (Cabodevilla et al., 2011b).

6.3.1 Methodological considerations

The methods used in this thesis were shown to be robust and in many circumstances replication of measurements such as development time, mortality and weight enabled comparisons between the data that informed results which in other circumstances may have appeared contradictory (Chapter 4). Smaller margins of measurement error could however have been obtained for the qPCR work. In the reported results, the method was shown to be reproducible and the level of virus detection replicable in over 70 different runs. Processing the samples in larger batches, for example by using 396-well reaction plates or a QIAcube to extract DNA from 96 insects simultaneously, is likely to reduce sample variability and the level of complexity in statistical models. This is important for larger trials such as the field study where processing batch often accounted for very large amounts of variation in comparison to biological effects (Appendices 4,5 and 6).

6.4 SUMMARY

In conclusion, these results provide novel insights into flight behaviour and the use of rotational flight mills, which can be used to test specific ecological theories and help interpret migratory infection dynamics in the field. The work presented here adds to the field of migratory disease ecology by providing the first evidence of Bateman's principle affecting flight tolerance of infection, specifically describing how this cost function is manifested as different flight behaviours in the two sexes. By manipulating the amount of time spent flying, these studies are also the first to quantify the relationship between flight effort and migratory tolerance as per Chapman et al. (2015b). The results from these studies reinforce different levels of disease tolerance in males and females. However, they also suggest that the response which drives differential life history strategies (Bateman's principle) is dynamic and possibly driven by seasonality or the level of sublethal infection in the background population. Finally, by investigating fluctuations of pathogen virulence over time and space in field populations, this work demonstrates how the cost functions observed in the laboratory relate to fluctuations in migratory infection dynamics in a relatively stable host-pathogen system.

APPENDIX 1: FLIGHT MILL STUDIES FROM 1961 TO 2016

Author: Melissa Minter, Rothamsted Research

Year	Author	Title	Journal	Notes	Species	Accuracy
1961	A.J. Cockbain	FUEL UTILIZATION AND DURATION OF TETHERED FLIGHT IN APHIS FABAE (SCOP)	J. Exp. Biol.	Flight duration only, tethered flight in flight chamber	Aphids	
1965	H. Dingle	THE RELATION BETWEEN AGE AND FLIGHT ACTIVITY IN THE MILKWEED BUG, *ONCOPELTUS*	J. Exp. Biol.	Analysed flight in the milkweed bug by testing on simple flight equipment and timed the flights. Dingle suggests the flight duration in the milkweed bug represents migration - longer flights represent migrants (ranging from 10 - >60 mins). Long flights represent persistent movement, one of Kennedy's (A turning point in the study of insect migration criteria for migration, Nature) Pattern of flight - began uneven but settled into a cruising phase a pattern similar to observed in Kennedy and Booth (1963) -aphids.	Milkweed bug, *Oncopeltus*	No - single measure - flight duration
1966	H. Dingle	SOME FACTORS AFFECTING FLIGHT ACTIVITY IN INDIVIDUAL MILKWEED BUGS	J. Exp. Biol.	Uses flight duration and mean flight duration in analyses. Uses flights over 30 mins, individuals which flew less than this were not included in study and were named 'non-flyers'.	Milkweed bug	Artificial cut off

1966	S. Vogel	FLIGHT IN DROSOPHILA .1. FLIGHT PERFORMANCE OF TETHERED FLIES	J. Exp. Biol.	Looks at speed, thrust, lift and angle in Drosophila flight.	Drosophila	
1974	Goldswor.G and W. Mordue	EFFECTS OF CORPUS CARDIACUM HORMONE ON TETHERED FLIGHT IN LOCUSTS	General and Comparative Endocrinology	No online access	Locust	
1979	G. J. Goldsworthy, A. R. Jutsum and N. L. Robinson	SUBSTRATE UTILIZATION AND FLIGHT SPEED DURING TETHERED FLIGHT IN THE LOCUST	Journal of Insect Physiology	Flight speed and flight time and substrate utilisation	Locust, *Corpora cardiaca*	
1980	M. A. Rankin and S. Rankin	SOME FACTORS AFFECTING PRESUMED MIGRATORY FLIGHT ACTIVITY OF THE CONVERGENT LADYBEETLE, *HIPPODAMIA-CONVERGENS* (COCCINELLIDAE, COLEOPTERA)	Biological Bulletin	Using tethered-flight technique to look for relationships between flight migratory behaviour in a Ladybird. Beetles that flew 30 mins nearly always continued to fly much longer. Thus, they determined that a performance of 30 mins was a reasonable indication of a migratory response, and used this factor for further analysis. "However, since Coleoptera usually move about by walking and fly only occasionally, the tendency to make long continuous flights is likely to indicate the tendency to migrate in this group (Southwood and Johnson, 1957; Southwood, 1962; Dingle, 1965)." Pg364	Ladybeetle, H*ippodamia-convergens*	No - artificial cut off
1980	M. A. Rankin	EFFECTS OF PRECOCENE I AND II ON FLIGHT BEHAVIOUR IN ONCOPELTUS FASCIATUS, THE MIGRATORY MILKWEED BUG	Journal of Insect Physiology	Assigned short flights as a few seconds to 3-4 mins and long flights one to several hours. Flights over 30min were defined as migratory as this was an indication of willingness to make longer flights. Most analyses used flights over 30min as a variable only.	Milkweed bug, *Oncopeltus fasciatus*	No - artificial cut off - only used flights over 30min ('migratory') for all analyses

1980	T. Ono and F. Nakasuji	COMPARISON OF FLIGHT ACTIVITY AND OVIPOSITION CHARACTERISTICS OF THE SEASONAL FORMS OF A MIGRATORY SKIPPER BUTTERFLY PARNARA-GUTTATA-GUTTATA	Kontyu	No online access	Skipper butterfly	
1980	F. Slansky	FOOD-CONSUMPTION AND REPRODUCTION AS AFFECTED BY TETHERED FLIGHT IN FEMALE MILKWEED BUGS (*ONCOPELTUS-FASCIATUS*)	Entomologia Experimentalis Et Applicata	No online access	Milkweed bugs (*Oncopeltus-fasciatus*)	
1980	A. G. Gatehouse and D. S. Hackett	A TECHNIQUE FOR STUDYING FLIGHT BEHAVIOR OF TETHERED *SPODOPTERA-EXEMPTA* MOTHS	Physiological Entomology	Take-off only	*Spodoptera-exempta*	
1981	P. S. Baker, M. Gewecke and R. J. Cooter	THE NATURAL FLIGHT OF THE MIGRATORY LOCUST, *LOCUSTA-MIGRATORIA* L WING-BEAT FREQUENCY, FLIGHT SPEED AND ATTITUDE	Journal of Comparative Physiology	No access online	Locust, *locusta-migratoria*	
1981	W. Kutsch and P. Stevenson	TIME-CORRELATED FLIGHTS OF JUVENILE AND MATURE LOCUSTS - A COMPARISON BETWEEN FREE AND TETHERED ANIMALS	Journal of Insect Physiology	No online access	Locusts	

1982	H. Dingle	FUNCTION OF MIGRATION IN THE SEASONAL SYNCHRONIZATION OF INSECTS	Entomologia Experimentalis Et Applicata	No online access	Insects	
1982	G. S. Pollack and N. Plourde	DIRECTIONALITY OF ACOUSTIC ORIENTATION IN FLYING CRICKETS	Journal of Comparative Physiology	No online access	Crickets	
1982	J. P. Ward and P. S. Baker	THE TETHERED FLIGHT PERFORMANCE OF A LABORATORY POPULATION OF *TRIATOMA INFESTANS* (KLUG) (HEMIPTERA: REDUVIIDAE)	Bulletin of Entomological Research	Uses flight durations against wing beat frequency variables	*Triatoma infestans*	
1984	M. A. Davis	THE FLIGHT AND MIGRATION ECOLOGY OF THE RED MILKWEED BEETLE (*TETRAOPES-TETRAOPHTHALMUS*)	Ecology	In analyses mean time to take off, time to take off, flight duration to represent flight performance in analyses that suggest migration and difference in flight potential between males & females.	Red Milkweed Beetle *Tetraopes tetraophthalmus*	
1984	G. S. Pollack, F. Huber and T. Weber	FREQUENCY AND TEMPORAL PATTERN-DEPENDENT PHONOTAXIS OF CRICKETS (*TELEOGRYLLUS-OCEANICUS*) DURING TETHERED FLIGHT AND COMPENSATED WALKING	Journal of Comparative Physiology	NA	Cricket, *Teleogryllus oceanicus*	

1985	W. E. Parker and A. G. Gatehouse	THE EFFECT OF LARVAL REARING CONDITIONS ON FLIGHT PERFORMANCE IN FEMALES OF THE AFRICAN ARMYWORM, *SPODOPTERA-EXEMPTA* (WALKER) (LEPIDOPTERA, NOCTUIDAE)	Bulletin of Entomological Research	Flights only greater that 6 min duration were used in analysis. To define long 'migratory' flight classified any moth with a total flight duration of >120 min in individual flights >30min during test period classified as long flier.	African armyworm, *Spodoptera exempta*	No - uses set flight duration to suggest migratory flight
1985	W. E. Parker and A. G. Gatehouse	GENETIC-FACTORS CONTROLLING FLIGHT PERFORMANCE AND MIGRATION IN THE AFRICAN ARMYWORM MOTH, *SPODOPTERA-EXEMPTA* (WALKER) (LEPIDOPTERA, NOCTUIDAE)	Bulletin of Entomological Research	Flights only greater that 6 min duration were used in analysis. To define long 'migratory' flight classified any moth with a total flight duration of >120 min in individual flights >30min during test period classified as long flier.	African armyworm, *Spodoptera exempta*	No - uses set flight duration to suggest migratory flight
1985	Z. Ruzicka and K. S. Hagen	IMPACT OF PARASITISM ON MIGRATORY FLIGHT PERFORMANCE IN FEMALES OF *HIPPODAMIA-CONVERGENS* (COLEOPTERA)	Acta Entomologica Bohemoslovaca	No online access	*Hippoedamia convergens* (Coleoptera)	
1986	M. L. McAnelly and M. A. Rankin	MIGRATION IN THE GRASSHOPPER *MELANOPLUS-SANGUINIPES* (FAB) .1. THE CAPACITY FOR FLIGHT IN NON-SWARMING POPULATIONS	Biological Bulletin	Both the number of "migrants" (defined as individuals which made at least one 60-minute flight) and the percentage of long flights made by "migrants" were determined.' (pg 373) Assessed differences in populations and thus migrants by the differences in short and intermediate flights (6-55min). They concluded that the short-term differences in environmental conditions between population habitats were not sufficient to account for the observed differences in migration. In discussion: **Long duration tethered flight has been correlated with evidence of migration in the field for several insect species (Dingle, 1965; Rose, 1972; Rankin and Rankin, 1980).**	Grasshopper *Melanoplus sanguinipes*	No - artificial cut off

1987	L. H. Teo, H. W. Fescemyer, J. P. Woodring and A. M. Hammond	CARBOHYDRATE AND FATTY-ACID TITERS DURING FLIGHT OF THE MIGRANT NOCTUID MOTH, *ANTICARSIA-GEMMATALIS* HUBNER	Insect Biochemistry	Uses flight time only in a comparison to fat and carb reserves.	Noctuid moth *Anticarsia gemmatalis*	Uses flight time only as flight variable - doesn't suggest migration
1987	K. P. Woodrow, A. G. Gatehouse and D. A. Davies	THE EFFECT OF LARVAL PHASE ON FLIGHT PERFORMANCE OF AFRICAN ARMYWORM MOTHS, *SPODOPTERA-EXEMPTA* (WALKER) (LEPIDOPTERA, NOCTUIDAE)	Bulletin of Entomological Research	Longest single flight, flight duration	African armyworm, *Spodoptera exempta*	
1989	A. Gunn, A. G. Gatehouse and K. P. Woodrow	TRADE-OFF BETWEEN FLIGHT AND REPRODUCTION IN THE AFRICAN ARMYWORM MOTH, *SPODOPTERA-EXEMPTA*	Physiological Entomology	Uses total flight duration and number of flights	African armyworm, *Spodoptera exempta*	
1990	J. C. A. Burchsted	ENHANCED REPRODUCTION FOLLOWING TETHERED FLIGHT IN THE MIGRATORY GRASSHOPPER, *MELANOPLUS-SANGUINIPES*	American Zoologist	No online access	Grasshopper, *Melanoplus Sanguinipes*	
1991	N. J. Armes and R. J. Cooter	EFFECTS OF AGE AND MATED STATUS ON FLIGHT POTENTIAL OF *HELICOVERPA-ARMIGERA*	Physiological Entomology	Mean flight duration, total flight, longest continuous flight, flight duration, flight speed	*Helicoverpa armigera*	

		(LEPIDOPTERA, NOCTUIDAE)				
1992	M. A. Rankin and J. C. A. Burchsted	THE COST OF MIGRATION IN INSECTS	Annual Review of Entomology	No online access		
1992	T. W. Sappington and W. B. Showers	LACK OF TRANSLATION OF DENSITY-INDUCED MORPHOLOGICAL POLYPHENISM TO LONG-DURATION FLIGHT BEHAVIOR OF BLACK CUTWORM (LEPIDOPTERA, NOCTUIDAE)	Annals of the Entomological Society of America	Only continuous flights of ^ 1 h were analysed to minimize the inclusion of trivial or appetitive flights (Gatehouse & Woodrow 1987). Variables of interest were duration (minutes) of the longest flight, time of flight initiation (minutes after dusk), time of flight termination (minutes after dusk), and flight speed (kilometres/hour)	Black cutworm, *Agrotis ipsilon*	
1993	E. N. Han and A. G. Gatehouse	FLIGHT CAPACITY - GENETIC DETERMINATION AND PHYSIOLOGICAL CONSTRAINTS IN A MIGRATORY MOTH *MYTHIMNA-SEPARATA*	Physiological Entomology	Total duration only to suggest migration syndrome through the proportion of 'long fliers'	Northern armyworm, *Mythimna separata*	No - flight duration only and suggestive migratory through 'long-fliers'
1993	A. Gunn and A. G. Gatehouse	THE MIGRATION SYNDROME IN THE AFRICAN ARMYWORM MOTH, *SPODOPTERA-EXEMPTA* - ALLOCATION OF RESOURCES TO FLIGHT AND REPRODUCTION	Physiological Entomology	Flight duration only -and resource allocation. Flight duration used in analyses to suggest migration.	African armyworm, *Spodoptera exempta*	No - only flight duration variable and suggests migratory in analyses

1993	I. Hodek, G. Iperti and M. Hodkova	LONG-DISTANCE FLIGHTS IN COCCINELLIDAE (COLEOPTERA)	European Journal of Entomology	Ladybirds perform three types of long distance flight and a 'trivial' flight assumed with foraging and ovipositing. Two types of long distance flight are associated with dormancy. Hagen (1962, 1966) discriminates between the directional migratory flight to and non-directional dispersal flight from dormancy sites. Data taken from other papers using tethered flight which use first flight, longest flight and total flight time (Ruzicka & Hagen, 1985)	Ladybirds	
1993	J. Colvin and A. G. Gatehouse	THE REPRODUCTION-FLIGHT SYNDROME AND THE INHERITANCE OF TETHERED-FLIGHT ACTIVITY IN THE COTTON-BOLLWORM MOTH, *HELIOTHIS-ARMIGERA*	Physiological Entomology	Longest flight time and total flight time and reproductive variables	*Helicoverpa armigera*	
1993	R. J. Cooter and N. J. Armes	TETHERED FLIGHT TECHNIQUE FOR MONITORING THE FLIGHT PERFORMANCE OF *HELICOVERPA-ARMIGERA* (LEPIDOPTERA, NOCTUIDAE)	Environmental Entomology	Uses number of flights, flying and resting times, initial speed, average speed,	*Helicoverpa armigera*	
1994	M. A. Rankin, E. N. Hampton and K. R. Summy	INVESTIGATIONS OF THE OOGENESIS-FLIGHT SYNDROME IN *ANTHONOMUS-GRANDIS* (COLEOPTERA, CURCULIONIDAE) USING TETHERED FLIGHT TESTS	Journal of Insect Behavior	Used % still flying, time in flight, mean flight time	*Anthonomus-grandis* (Coleoptera, Curculionidae	
1995	T. W. Sappington, H. W. Fescemyer and W. B. Showers	LIPID AND CARBOHYDRATE UTILIZATION DURING FLIGHT OF THE MIGRATORY MOTH, *AGROTIS-IPSILON*	Archives of Insect Biochemistry and Physiology	Used flight duration only as flight variable. Classified flights as short-fliers (>1h and <5h) and long-fliers (>5h).	Black cutworm, *Agrotis-ipsilon*	No - used only flight duration. Cut-off between

		(LEPIDOPTERA, NOCTUIDAE)				fliers and non-fliers
1995	A. C. Nilssen and J. R. Anderson	FLIGHT CAPACITY OF THE REINDEER WARBLE FLY, *HYPODERMA-TARANDI* (L), AND THE REINDEER NOSE BOT FLY, *CEPHENEMYIA-TROMPE* (MODEER) (DIPTERA, OESTRIDAE)	Canadian Journal of Zoology-Revue Canadienne De Zoologie	No online access	Reindeer nose bot fly, *Cephenemyia trompe*	
1995	L. G. Luo Lizhi and Y. Hu	RELATIONSHIP BETWEEN FLIGHT CAPACITY AND OVIPOSITION OF ORIENTAL ARMYWORM MOTHS, *MYTHIMNA SEPARATA* (WALKER)	Acta Entomologica Sinica	Not in English	Oriental armyworm, *Mythimna separata*	
1995	Y. Shirai	LONGEVITY, FLIGHT ABILITY AND REPRODUCTIVE PERFORMANCE OF THE DIAMONDBACK MOTH, *PLUTELLA XYLOSTELLA* (L) (LEPIDOPTERA: YPONOMEUTIDAE), RELATED TO ADULT BODY SIZE	Researches on Population Ecology	No online access	Diamond back moth, *Plutella xylostella*	
1995	M. Lorez	NEURAL CONTROL OF HINDLEG STEERING IN-FLIGHT IN THE LOCUST	Journal of Experimental Biology	Steering and neural control.		

1995	K. R. Beerwinkle, J. D. Lopez, D. Cheng, P. D. Lingren and R. W. Meola	FLIGHT POTENTIAL OF FERAL *HELICOVERPA-ZEA* (LEPIDOPTERA, NOCTUIDAE) MALES MEASURED WITH A 32-CHANNEL, COMPUTER-MONITORED, FLIGHT-MILL SYSTEM	Environmental Entomology	Time of 1st flight take off, number of flights, total distance of all flights, distance of longest single flight, duration of all flights, duration of longest single flight, speed during all flights, speed during longest single flight.	*Helicoverpa zea*	
1995	J. Colvin	THE REGULATION OF MIGRATION IN *HELICOVERPA ARMIGERA*	Insect Migration: Tracking Resources through Space and Time	Review of other studies	*Helicoverpa armigera*	
1996	D Weber and D Ferro	FLIGHT AND FECUNDITY OF COLORADO POTATO BEETLES (COLEOPTERA: CHRYSOMELIDAE) FED ON DIFFERENT DIET	Behaviour	Use mean flight duration and total flight only	Colorado Potato Beetles (Coleoptera: Chrysomelidae)	
1996	D. R. Horton and T. M. Lewis	TETHERED FLIGHT ACTIVITY OF PEAR PSYLLA, CACOPSYLLA PYRICOLA: SEASONAL, HOST, AND MORPHOTYPIC EFFECTS	Entomologia Experimentalis Et Applicata	Using minutes in flight and max flight duration and morphological effects.	Pear psylla, *Cacopsylla pyricola*	
1997	J. W. Kent, Jr., Y. Teng, D. Deshpande, M. A. Rankin and Y. M. Teng	MOBILIZATION OF LIPID AND CARBOHYDRATE RESERVES IN THE MIGRATORY GRASSHOPPER *MELANOPLUS SANGUINIPES*	Physiological Entomology	In this analyses assigned presumed migrants as (made at least two 1 h tethered flights) or non-migrants (flew no longer than 30 min in any trial). They stated that individuals which fly for at least 60 min will continue to fly for much longer periods. An individual that does not perform at least one 60min flight is unlikely to make a long flight at any time. Only used duration as a flight performance measure to compare to other elements and to assign 'presumed migrants' and 'non-migrants'.	Grasshopper *Melanoplus sanguinipes*	No - artificial cut offs

1997	P. Schumacher, A. Weyeneth, D. C. Weber and S. Dorn	LONG FLIGHTS IN *CYDIA POMONELLA* L (LEPIDOPTERA: TORTRICIDAE) MEASURED BY A FLIGHT MILL: INFLUENCE OF SEX, MATED STATUS AND AGE	Physiological Entomology	Flight capacity - longest single flight and total distance flown. Take-off frequency and average velocity is also used. Separated 'long fliers' as more than 6km in longest single and 'short fliers' as less than 6km in longest single flight. Based on understanding dispersal	*Cydia pomonella L.* (Lepidoptera)	Used various flight variables but cut off short fliers from long fliers using the longest single flight variable.
1997	P. A. Stevenson	REFLEX ACTIVATION OF LOCUST FLIGHT MOTONEURONES BY PROPRIOCEPTORS RESPONSIVE TO MUSCLE CONTRACTIONS	Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology	Flight muscle waves	Locust	
1997	M. Coombs	TETHERED-FLIGHT AND AGE-RELATED REPRODUCTIVE PERFORMANCE OF *HELICOVERPA PUNCTIGERA* (WALLENGREN) AND *H-ARMIGERA* (HUBNER) (LEPIDOPTERA: NOCTUIDAE)	Australian Journal of Zoology	No online access	*Helicoverpa punctigera* and *H-armigera*	
1997	J. R. Riley, M. C. A. Downham and R. J. Cooter	COMPARISON OF THE PERFORMANCE OF CICADULINA LEAFHOPPERS ON FLIGHT MILLS WITH THAT TO BE EXPECTED IN FREE FLIGHT	Entomologia Experimentalis Et Applicata	No online access	Cicadulina leafhoppers	

1998	M. C. A. Downham and R. J. Cooter	TETHERED FLIGHT AND MORPHOMETRIC STUDIES WITH *CICADULINA STOREYI* AND *C-MBILA* LEAFHOPPERS (HEMIPTERA: CICADELLIDAE) VECTORS OF MAIZE STREAK VIRUS IN UGANDA	Bulletin of Entomological Research	Flight durations, duration of total flight	*Cicadulina storeyi* and *C mbila* leafhoppers	No - flight variable duration only
1998	L. Auerswald, P. Schneider and G. Gade	UTILISATION OF SUBSTRATES DURING TETHERED FLIGHT WITH AND WITHOUT LIFT GENERATION IN THE AFRICAN FRUIT BEETLE *PACHNODA SINUATA* (CETONIINAE)	Journal of Experimental Biology	A study analysing the use of substrates during flight in the African fruit beetle. Using flight duration and duration of rest after 5 mins of flight. Also used flight velocity as a measure of performance during flight.	African fruit beetle *Pachnoda sinuata*	

1998	C. E. Gee, K. L. Shoemaker and R. M. Robertson	THE FOREWING TEGULAE: THEIR SIGNIFICANCE IN STEERING MANOEUVRES AND FREE FLIGHT IN LOCUSTA MIGRATORIA	Canadian Journal of Zoology-Revue Canadienne De Zoologie	No online access	Locust, *Locusta migratoria*	
1998	B. Hedwig and G. Becher	FOREWING MOVEMENTS AND INTRACELLULAR MOTONEURONE STIMULATION IN TETHERED FLYING LOCUSTS	Journal of Experimental Biology	Wing movements, frequencies etc.	Locusts	
1999	F. O. Lehmann	AMBIENT TEMPERATURE AFFECTS FREE-FLIGHT PERFORMANCE IN THE FRUIT FLY *DROSOPHILA MELANOGASTER*	Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology	No online access	fruit fly *Drosophila melanogaster*	
1999	L. Luo, X. Jiang, K. Li and Y. Hu	INFLUENCES OF FLIGHT ON REPRODUCTION AND LONGEVITY OF THE ORIENTAL ARMYWORM, MYTHIMNA SEPARATA (WALKER)	Acta Entomologica Sinica	Not in English	oriental armyworm, *Mythimna separata*	
2000	Y Shirai and Y Kosugi	FLIGHT ACTIVITY OF THE SMALLER TEA TORTRIX,	App. Entomol. Zool.	Assessed flight activity using flight duration and flight velocity and used continuous flight.	Tea tortrix (moth)	
2000	R. J. Cooter, D. Winder and T. C. B. Chancellor	TETHERED FLIGHT ACTIVITY OF *NEPHOTETTIX VIRESCENS* (HEMIPTERA: CICADELLIDAE) IN THE PHILIPPINES	Bulletin of Entomological Research	No online access	Leafhopper *Nephotettix virescens*	

2000	Vogt J, Appel, A and West M	FLIGHT ENERGETICS AND DISPERSAL CAPABILITY OF THE FIRE ANT, *SOLENOPSIS INVICTA* BUREN	Journal of Insect Physiology	Total flight duration and flight speed to conclude that flight capability of *S. invicta* female alates is limited to <5 km	fire ant, *Solenopsis invicta*	
2001	J. W. Kent and M. A. Rankin	HERITABILITY AND PHYSIOLOGICAL CORRELATES OF MIGRATORY TENDENCY IN THE GRASSHOPPER *MELANOPLUS SANGUINIPES*	Physiological Entomology	In results of this paper - individuals that flew for at least 60 min were classified as migrants, and those that flew less than 10 mins were non-migrants. Used time in flight (m) for analyses and stated that if an individual made one 60min flight it was 'likely' to make another.	grasshopper *Melanoplus sanguinipes*	No - artificial cut offs
2001	Kent and Rankin	HERITABILITY AND PHYSIOLOGICAL CORRELATES OF MIGRATORY TENDENCY IN THE GRASSHOPPER *MELANOPLUS SANGUINIPES*	Physiological Entomology	Migratory flight of north American grasshopper - using flight duration as a measure. They concluded that most individuals will not fly, or will fly for many hours. They suggested a measure of the 'one-hour rule' for distinguishing migrants from non-migrants - very crude measure. Fig 2 shows flight records where they suggest that if an individual made a 60-min flight they were 'likely' to make another - but not secure evidence to back this up (only estimates of repeatability table 1). "*Melanoplus sanguinipes* populations exhibit a bimodal distribution of migratory behaviour: individuals tend either to not fly (or make very short flights) or to fly > 1 h. Individuals that fly > 1 h are likely to do so again if the appropriate cues are present, although they may not fly at every opportunity. As noted earlier, this is the basis for McAnelly's (1985) one-hour rule for identifying presumed migrants and non-migrants" (Discussion, page 378)	grasshopper *Melanoplus sanguinipes*	No - cut off and single flight variable (flight duration)

2001	C. Feng, B. Zhai, X. Zhang, C. H. Feng, B. P. Zhai and X. X. Zhang	RE-EMIGRATION CAPACITY OF THE BROWN PLANTHOPPER, *NILAPARVATA LUGENS*	Chinese Journal of Rice Science	No online access	Brown planthopper, *Nilaparvata lugens*	
2001	B. S. Wu, V. K. Walker and R. M. Robertson	HEAT SHOCK-INDUCED THERMOPROTECTION OF ACTION POTENTIALS IN THE LOCUST FLIGHT SYSTEM	Journal of Neurobiology	No online access	Locust	
2001	Yamanaka T, Tatsuki S and Shimada M	FLIGHT CHARACTERISTICS AND DISPERSAL PATTERNS OF FALL WEBWORM (LEPIDOPTERA: ARCTIIDAE) MALES	Environmental entomology	Use flight velocity, flight duration and flight distance to understand dispersal	Fall webworm (Lepidoptera: Arctiidae)	
2002	M. Murata and S. Tojo	UTILIZATION OF LIPID FOR FLIGHT AND REPRODUCTION IN *SPODOPTERA LITURA* (LEPIDOPTERA: NOCTUIDAE)	European Journal of Entomology	Flight duration only as flight parameter in comparison to lipid reserves.	*Spodoptera litura*	
2002	H. Mouritsen and B. J. Frost	VIRTUAL MIGRATION IN TETHERED FLYING MONARCH BUTTERFLIES REVEALS THEIR ORIENTATION MECHANISMS	Proceedings of the National Academy of Sciences of the United States of America	Orientation	Monarch butterfly	

2002	L. Z. Luo, S. J. Johnson, A. M. Hammond, J. D. Lopez, J. P. Geaghan, K. R. Beerwinkle and J. K. Westbrook	DETERMINATION AND CONSIDERATION OF FLIGHT POTENTIAL IN A LABORATORY POPULATION OF TRUE ARMYWORM (LEPIDOPTERA: NOCTUIDAE)	Environmental Entomology	Used longest flight duration, total flight duration, total flight distance and average flight speed to conclude that migrant *P. unipuncta* moth possess great flight potential, with long flights.	True armyworm	
2002	H. Fischer, H. Wolf and A. Buschges	THE LOCUST TEGULA: KINEMATIC PARAMETERS AND ACTIVITY PATTERN DURING THE WING STROKE	Journal of Experimental Biology	Wing stoke, burst duration, down stroke interval (wing parameters)	Locust	
2003	C MacQuarrie and G Boiteau	EFFECT OF DIET AND FEEDING HISTORY ON FLIGHT OF COLORADO POTATO BEETLE	Entomologia Experimentalis et Applicata	Use mean flight frequency	Colorado potato beetle	
2004	K. J. Min, N. Jones, D. W. Borst and M. A. Rankin	INCREASED JUVENILE HORMONE LEVELS AFTER LONG-DURATION FLIGHT IN THE GRASSHOPPER, *MELANOPLUS SANGUINIPES*	Journal of Insect Physiology	Used one hour flights and 'flight to exhaustion'. Classifying flights of 60 minutes as presumed migrants (McAnelly 1985). This study compares insects which have made 1hr flights to those have flown to exhaustion and those which refused to make a long flight - and assigning these as migrants and non-migrants.	Grasshopper, *Melanoplus sanguinipes*	No - cut off and presumption of migrants with lack of parameters
2004	K. J. Min, T. E. Taub-Montemayor, K. D. Linse, J. W. Kent and M. A. Rankin	RELATIONSHIP OF ADIPOKINETIC HORMONE I AND II TO MIGRATORY PROPENSITY IN THE GRASSHOPPER, *MELANOPLUS SANGUINIPES*	Archives of Insect Biochemistry and Physiology	Uses flight duration only as a parameter and assigning 'long fliers' as migrants	Grasshopper, *Melanoplus sanguinipes*	No - single parameter

2004	J. L. Blackmer, S. E. Naranjo and L. H. Williams	TETHERED AND UNTETHERED FLIGHT BY *LYGUS HESPERUS* AND *LYGUS LINEOLARIS* (HETEROPTERA: MIRIDAE)	Environmental Entomology	Analyses involved flying individuals over a 23-hour period and the variables of number of flights, mean duration and cumulative duration were measured and compared to species, sex and age to assess flight performance.	*Lygus hesperus* and *Lygus lineolaris* (bugs)	
2004	M. Murata and S. Tojo	FLIGHT CAPABILITY AND FATTY ACID LEVEL IN TRIACYLGLYCEROL OF LONG-DISTANCE MIGRATORY ADULTS OF THE COMMON CUTWORM, *SPODOPTERA LITURA*	Zoological Science	Flight duration only as flight parameter in comparison to lipid reserves.	Spodoptera litura	
2004	Y.-K. Wang and B.-P. Zhai	RE-MIGRATION CAPACITY OF THE WHITE-BACKED PLANTHOPPER, *SOGATELLA FURCIFERA* (HORVATH)	Acta Entomologica Sinica	Not in English	White-backed planthopper, *Sogatella furcifera*	

2004	Y Ishiguri and Y Shirai	FLIGHT ACTIVITY OF THE PEACH FRUIT MOTH, *CARPOSINA SASAKII* (LEPIDOPTERA: CARPOSINIDAE), MEASURED BY A FLIGHT MILL. APPL.	App. Entomol. Zool.	Used flight speed, total flight duration, % of non-fliers, % of long fliers	peach fruit moth, *Carposina sasakii*	
2005	J. A. Stebbing, L. J. Meinke, S. E. Naranjo, B. D. Siegfried, R. J. Wright and L. D. Chandler	FLIGHT BEHAVIOR OF METHYL-PARATHION-RESISTANT AND -SUSCEPTIBLE WESTERN CORN ROOTWORM (COLEOPTERA: CHRYSOMELIDAE) POPULATIONS FROM NEBRASKA	Journal of Economic Entomology	No online access	corn rootworm (Coleoptera: Chrysomelidae	
2005	K.-B. Li, Y.-Z. Cao, L.-Z. Luo, X.-W. Gao, J. Yin and Y. Hu	INFLUENCES OF FLIGHT ON ENERGETIC RESERVES AND JUVENILE HORMONE SYNTHESIS BY CORPORA ALLATA OF THE ORIENTAL ARMYWORM, MYTHIMNA SEPARATA (WALKER)	Acta Entomologica Sinica	Not in English		
2006	H Wanner, H Gu and S Dorn	NUTRITIONAL VALUE OF FLORAL NECTAR SOURCES FOR FLIGHT INTHE PARASITOID WASP, *COTESIA GLOMERATA*	Physiological Entomology	Comparison of flight capacity in *Cotesia glomerata* under different feeding regimes using total distance flown (m), number of flights, longest single flight (s), Average flight velocity.	Parasitoid wasp, *Cotesia glomerata*	
2007	S. P. Sane, A. Dieudonne, M. A. Willis and T. L. Daniel	ANTENNAL MECHANOSENSORS MEDIATE FLIGHT CONTROL IN MOTHS	Science	Flight dynamics including collisions, wing beat	Moths	

2008	M. Saastamoinen and I. Hanski	GENOTYPIC AND ENVIRONMENTAL EFFECTS ON FLIGHT ACTIVITY AND OVIPOSITION IN THE GLANVILLE FRITILLARY BUTTERFLY	The American naturalist	No online access	Butterfly	
2008	D. L. Dorhout, T. W. Sappington and M. E. Rice	EVIDENCE FOR OBLIGATE MIGRATORY FLIGHT BEHAVIOR IN YOUNG EUROPEAN CORN BORER (LEPIDOPTERA: CRAMBIDAE) FEMALES	Environmental Entomology	Uses duration of longest flight, minimum duration of longest single flight, total flight duration, speed during longest single flight.	Moth European Corn Borer, Ostrinia nubilalis	
2008	L. Han, H. Gu, B. Zhai, X. Zhang, L. Z. Han, H. N. Gu, B. P. Zhai and X. X. Zhang	REPRODUCTION-FLIGHT RELATIONSHIP IN THE BEET ARMYWORM, SPODOPTERA EXIGUA (LEPIDOPTERA: NOCTUIDAE)	Environmental Entomology	Uses mean flight duration, mean flight distance and mean flight speed to compare to show relationship between flight capacity and reproduction.	Beet armyworm, Spodoptera exigua	

2009	O. A. Bruzzone, J. M. Villacide, C. Bernstein and J. C. Corley	FLIGHT VARIABILITY IN THE WOODWASP *SIREX NOCTILIO* (HYMENOPTERA: SIRICIDAE): AN ANALYSIS OF FLIGHT DATA USING WAVELETS	Journal of Experimental Biology	"Although flight mill experiments are common in insect dispersal studies, data analyses have focused mostly on the main characteristic of flight. Typically, total distance travelled, total flight duration, and average flight speed are measured (e.g. Weber and Ferro, 1996; Vogt et al., 2000; Yamanaka et al., 2001; Luo et al., 2002). Occasionally, other characteristics related to the continuity of the flight, such as the extension of continuous flight, or flight interruptions, the length of pulses or pauses between successive flights have also been quantified (**Shirai and Kosugi, 2000; Ishiguri and Shirai, 2004; MacQuarrie and Boitau, 2003; Blackmer et al., 2004; Wanner et al., 2006**)"'However, because the insects are forced to fly in a flight mill as a result of permanent stimulation by lack of tarsal contact (Edwards, 2006), classical analysis of flight mill data tend to overestimate the flight distance and the total flight time by a factor of ten or more, compared with other laboratory studies such as those carried out in flight chambers (Shirai and Kosugi, 2000; Yamanaka et al., 2001; Blackmer et al., 2004) or field methods such as mark–release–recapture (Botero-Garces and Isaacs, 2004). To our knowledge, a detailed exploration of the complete data set obtained from tethered flight studies.'In this study wavelet analyses (presented as 3D contour plots in used to analyse flight in the woodwasp. Classified 3 flights: - regular - a single, long sustained flight with a changing but slowing speed with slight acceleration at first and steady slowdown after reaching max flight speed. Periodic - long sustained flight without resting but periodically variable speed. Flight span between two pauses was longer than an hour. Pulsating flight- short pulses of flights followed by longer pauses.	Woodwasp, *Sirex noctilio*	

2010	Y.-G. Tu, K.-M. Wu, F.-S. Xue and Y.-H. Lu	LABORATORY EVALUATION OF FLIGHT ACTIVITY OF THE COMMON CUTWORM, *SPODOPTERA LITURA* (LEPIDOPTERA: NOCTUIDAE)	Insect Science	Flight variables used total flight duration, total flight distance, average light speed	Common cutworm, *Spodoptera litura*	
2010	X. F. Jiang, L. Z. Luo and T. W. Sappington	RELATIONSHIP OF FLIGHT AND REPRODUCTION IN BEET ARMYWORM, *SPODOPTERA EXIGUA* (LEPIDOPTERA: NOCTUIDAE), A MIGRANT LACKING THE OOGENESIS-FLIGHT SYNDROME	Journal of Insect Physiology	In analyses use flight duration, flight velocity, and flight distance - this along with reproductive traits they used to show migration	beet armyworm, *Spodoptera exigua*	
2010	H. Kong, L. Luo, X. Jiang and L. Zhang	EFFECTS OF LARVAL DENSITY ON FLIGHT POTENTIAL OF THE BEET WEBWORM, LOXOSTEGE STICTICALIS (LEPIDOPTERA: PYRALIDAE)	Environmental Entomology	Looks at the effect of rearing density on flight behaviour of Beet webworm using flight parameters including total flight distance, flight velocity, total flight distance, longest flight duration.	Beet webworm	

2010	Y.-B. Gao and B.-P. Zhai	ACTIVE TEMPERATURE SELECTION OF FLYING *HELICOVERPA ARMIGERA* (LEPIDOPTERA: NOCTUIDAE) MOTHS	Acta Entomologica Sinica	Not in English	*Helicoverpa armigera*	
2010	S. Jiang, L. Luo, Y. Hu, L. Zhang, S. J. Jiang, L. Z. Luo, Y. Hu and L. Zhang	EFFECTS OF CRY1AC PROTEIN ON GROWTH AND DEVELOPMENT, REPRODUCTION AND FLIGHT POTENTIAL OF THE ORIENTAL ARMY WORM, MYTHIMNA SEPARATA (LEPIDOPTERA: NOCTUIDAE)	Acta Entomologica Sinica	Not in English	Oriental armyworm, *Mythimna separata*	
2010	F. Wang, X. Zhang, B. Zhai, F. Y. Wang, X. X. Zhang and B. P. Zhai	FLIGHT AND RE-MIGRATION CAPACITY OF THE RICE LEAFFOLDER MOTH, *CNAPHALOCROCIS MEDINALIS* (GUENEE) (LEPIDOPTERA: CRAMBIDAE)	Acta Entomologica Sinica	Not in English	Rice leaffolder moth, *Cnaphalocrocis medinalis*	

2010	R. A. J. Taylor, L. S. Bauer, T. M. Poland and K. N. Windell	FLIGHT PERFORMANCE OF *AGRILUS PLANIPENNIS* (COLEOPTERA: BUPRESTIDAE) ON A FLIGHT MILL AND IN FREE FLIGHT	Journal of Insect Behavior	Uses flight variables: The number of laps flown, the distance (= laps * 57 cm circumference),duration, and the average flight speed (= distance/duration) of each individual flight bout; 2. The mean and variance of number of laps/bout, distance flown/bout, duration/bout, and flight speed/bout for each individual insect; 3. The total distance flown, the total time spent flying, and the total time spent resting by each individual insect; 4. The sample means and variances for all variables across all insects in the run.	Ash borer *Agrilus planipennis* (Coleoptera: Buprestidae)	
2011	J. Liu, J. Cui, Y. Zhang, T. Deng, X. Zhao, J. Q. Liu, J. X. Cui, Y. Q. Zhang, T. F. Deng and X. D. Zhao	COMPARISON OF SEX-SPECIFIC TETHERED FLIGHT OF MUSCA DOMESTICA	Zhongguo Meijie Shengwuxue ji Kongzhi Zazhi = Chinese Journal of Vector Biology and Control	No online access	House flies	
2012	Y. X. Cheng, L. Z. Luo, X. F. Jiang and T. W. Sappington	SYNCHRONIZED OVIPOSITION TRIGGERED BY MIGRATORY FLIGHT INTENSIFIES LARVAL OUTBREAKS OF BEET WEBWORM	Plos One	Only uses flight distance as a flight performance proxy and compares to oviposition to suggest migration in the beet webworm. Analyses on based on 'migratory' individuals which have been established through flight distance.	Beetwebworm, *Spoladea recurvalis*	No - flight duration only to suggest migratory individuals

2012	C. G. Elliott and M. L. Evenden	THE EFFECT OF FLIGHT ON REPRODUCTION IN AN OUTBREAKING FOREST LEPIDOPTERAN	Physiological Entomology	Analysing the effect of flight on reproduction using flight duration against reproductive traits.	Moth *Choristoneura conflictana*	No - flight variable duration only
2012	E. P. Snelling, R. S. Seymour, P. G. D. Matthews and C. R. White	MAXIMUM METABOLIC RATE, RELATIVE LIFT, WINGBEAT FREQUENCY AND STROKE AMPLITUDE DURING TETHERED FLIGHT IN THE ADULT LOCUST *LOCUSTA MIGRATORIA*	Journal of Experimental Biology	Wing beat frequency and stroke amplitude	*Locusta migratoria*	
2013	N. Ding, X. Xin, X. Zhou, X. Men, Y. Yu, A. Zhang, L. Li, Q. Zhuang, N. Ding, X. G. Xin, X. H. Zhou, X. Y. Men, Y. Yu, A. S. Zhang, L. L. Li and Q. Y. Zhuang	EFFECTS OF BODY WEIGHT AND FEEDING ON THE FLIGHT CAPACITY OF ADULTS OF *OSMIA EXCAVATA* (HYMENOPTERA: MEGACHILIDAE)	Acta Entomologica Sinica	Not in English	Bee, *Osmia excavata*	
2014	A. Krishnan and S. P. Sane	VISUAL FEEDBACK INFLUENCES ANTENNAL POSITIONING IN FLYING HAWK MOTHS	Journal of Experimental Biology	Flight angles not parameters.	Hawk moths	

2014	L. A. Castro, J. K. Peterson, A. Saldana, M. Y. Perea, J. E. Calzada, V. Pineda, A. P. Dobson and N. L. Gottdenker	FLIGHT BEHAVIOR AND PERFORMANCE OF *RHODNIUS PALLESCENS* (HEMIPTERA: REDUVIIDAE) ON A TETHERED FLIGHT MILL	Journal of Medical Entomology	In analyses used: Flight initiation measurements were: 1) type and number of stimuli provoking flight initiation, 2) time to first flight initiation, and 3) time to first sustained flight bout. Flight performance parameters were 1) endurance, 2) speed, and 3) total number of flights per insect. Endurance measurements were 1) cumulative flight time across all flights, 2) cumulative distance flown across all flights, 3) longest distance and time flown in a single flight bout without resting, and 4) average distance and time flown per insect across all flights.	*Rhodnius pallescens* (Bug)	
2014	LuWeiXiang, X. Jiang, L. Zhang, L. Luo, W. X. Lu, X. F. Jiang, L. Zhang and L. Z. Luo	EFFECT OF DIFFERENT TETHERED FLIGHT DURATIONS ON THE REPRODUCTION AND ADULT LONGEVITY OF *MYTHIMNA SEPARATA* (LEPIDOPTERA: NOCTUIDAE)	Chinese Journal of Applied Entomology	No online access	Oriental armyworm, *Mythimna separata*	
2014	V. M. Lopez, M. N. McClanahan, L. Graham and M. S. Hoddle	ASSESSING THE FLIGHT CAPABILITIES OF THE GOLDSPOTTED OAK BORER (COLEOPTERA: BUPRESTIDAE) WITH COMPUTERIZED FLIGHT MILLS	Journal of Economic Entomology	Use average total flight distance, average total flight duration, average velocity, total number of flight bouts, average flight bout time	Goldspotted Oak Borer, Jewel beetle	

2015	A. Attisano, J. T. Murphy, A. Vickers and P. J. Moore	A SIMPLE FLIGHT MILL FOR THE STUDY OF TETHERED FLIGHT IN INSECTS	Jove-Journal of Visualized Experiments	Includes protocol of creating and running flight mill - little on output of data which is all concentrated on speed (m/s). Using flight data from milkweed bug they observed two patterns of behaviour A) a typical flight of a migrant - flying at steady speed over long periods of times B) flight of a resident - flying at lower speeds and flight bouts only last for short time. https://www.jove.com/files/ftp_upload/53377/53377fig6large.jpg	Insects	
2015	X. Jiang, L. Zhang, Y. Cheng, L. Luo, X. F. Jiang, L. Zhang, Y. X. Cheng and L. Z. Luo	CHANGES OF BODY TEMPERATURE AND RESPIRATION RATE DURING FIXED FLIGHT OF THE ORIENTAL ARMY WORM, MYTHIMNA SEPARATA (WALKER)	Acta Phytophylacica Sinica	Not in English	Oriental army worm, *Mythimna separata*	
2015	R. Yuan, X. Wang, S. Yang, P. Chen, R. L. Yuan, X. W. Wang, S. Yang and P. Chen	CHANGES IN THE ACTIVITIES OF ENZYMES RELATED TO ENERGY METABOLISM IN FLIGHT MUSCLES OF ADULT *BACTROCERA DORSALIS* (DIPTERA: TEPHRITIDAE) AT DIFFERENT AGES AND DURING TETHERED FLIGHT	Acta Entomologica Sinica	No online access	*Bactrocera dorsalis* (Diptera: Tephritidae)	
2015	J. Caballero, C. Mazo, I. Rodriguez-Pinto and J. C. Theobald	A VISUAL HORIZON AFFECTS STEERING RESPONSES DURING FLIGHT IN FRUIT FLIES	Journal of Experimental Biology	Tethered flight to analyse elevation and angle of flying fruit flies.	Fruit flies Drosophila	
2015	S. Sadaf, O. V. Reddy, S. P.	NEURAL CONTROL OF WING COORDINATION IN FLIES	Current Biology	Wing beat frequency, amplitude etc.	Flies	

	Sane and G. Hasan					
2015	S. Mureli and J. L. Fox	HALTERE MECHANOSENSORY INFLUENCE ON TETHERED FLIGHT BEHAVIOR IN DROSOPHILA	Journal of Experimental Biology	Wing beat frequency	Fruit fly Drosophila	
2015	T. Deora, A. K. Singh and S. P. Sane	BIOMECHANICAL BASIS OF WING AND HALTERE COORDINATION IN FLIES	Proceedings of the National Academy of Sciences of the United States of America	Wing beat, frequency, positioning of wings	Flies	
2016	H. B. C. Jones, K. S. Lim, J. R. Bell, J. K. Hill and J. W. Chapman	QUANTIFYING INTERSPECIFIC VARIATION IN DISPERSAL ABILITY OF NOCTUID MOTHS USING AN ADVANCED TETHERED FLIGHT TECHNIQUE	Ecology and Evolution	Total distance, total duration, number of flights, average flight distance, average flight duration, average flight speed, max speed, Ffdistance, Ffduration, Ffavspeed, Ffmaxspeed, furthest flight distance, longest flight distance, long flight duration, longest flight average speed, longest flight max speed. Principal component analysis of the 16 flight variables.	Noctuid moths	

APPENDIX 2: PROTOCOLS

REARING PROTOCOLS FOR *S. EXEMPTA* AND *S. FRUGIPERDA*

Authors

Aislinn Pearson and Kenneth Wilson (*Spodoptera frugiperda and S. exempta*

NB: Always work with *S. exempta* first to avoid contamination of gregarious cultures with *S. frugiperda* larvae, as *S. frugiperda* is cannibalistic and will consume all the exempta larvae in a rearing pot. The only exception to this is when there is a problem with fungus in the exempta diet.

EGGS:

Every other day:

- Remove and replace tissue paper and nappy liners.
- Cut 12 egg batches (6 from each cage) and place in a polypot with diet. Label each polypot with a unique family ID. Place the egg batches from in a tray labelled 'EGGS' with the species and generation.
- When you have collected 48 egg batches (approximately 8 days after adults started laying) freeze the mating cages. Remove from the freezer after 72 hours and clean thoroughly.

LARVAE:

Daily:

- Check egg batches daily and collect any pots with neonate larvae. Place polypots with neonate larvae in a new tray labelled with the species, generation and hatching date written 'Day of the week DD.MM.YYYY'.
- Record the date of emergence in the rearing records

48 hours after emergence:

- On the third day after emergence, thin the following number of larvae from each family as follows:

  Spodoptera exempta: Line 354ml pots with a piece of filter paper and place a strip of diet on the filter paper. Set up one pot per family. Take 30 – 35 larvae from each family and

place them in the diet pot. Pierce the lid with several holes and line with blue roll to help regulate humidity and stop early instar larvae escaping (late instar larvae may eat the blue roll – this is not a problem).

Spodoptera frugiperda: Take 5 larvae from each family and place each larva in an individual 37ml polypot (S. frugiperda are cannibalistic and cannot be cultured gregariously as with exempta). Again, pierce the lid (two holes is ample) and line with blue roll

- Record the date and number thinned in the rearing records.
- Once thinned, place the pots back in the tray, and add the thinning day and date to the label ('T1: day of the week DD.MM.YY)

Five days after thinning (*S. exempta* only):

- Take 6 larvae from each 350ml tub and transfer to individual polypots lined with blue roll.
- Record the day, date and number thinned from each polypot on the rearing records. Make a note if there are any virus deaths in the pot or if there is a prolific problem with mould.
- Label the tray with the date of thinning (T2: Day of the week DD.MM.YYY)

NB: Before moving insects to a new pot, set out relevant number of diet pots and allow them to stand for about 15 minutes before introducing the larvae. This allows any excess water to evaporate and helps control fungal contamination of the diet. Early instar larvae are also prone drowning in the excess water.

Daily after thinning:

- Check larvae regularly (daily for exempta and every other day for frugiperda).
- Throw away any insects which are dead or dying – this is particularly important for the exempta cultures.
- Replace any diet that has become mouldy.
- If the S. exempta culture has become contaminated with S. frugiperda (or vice versa) remove the erroneous species. The two species are very easy to tell apart from the 3rd instar:

S. frugiperda tends to be brown and has raised black protrusions or "lumps" on its skin, which is unlike any other *Spodoptera* species.

S. exempta lacks these black ridges and is **often uniformly black** after being reared at high density, although it may also be green with lateral stripes.

S. frugiperda *S. exempta*

H. armigera

NB: Always clean down the surfaces with Distel when moving between cultures to prevent contamination

PUPAE & ADULTS

Every other day:

- Check cultures for pupae. Where insects have pupated, collect individuals from the same family into a 37ml polypot lined with vermiculite.
- Once the majority of the insects in a tray have become pupae, set up the mating cages. given tray have pupated, set up the mating cages as follows:
    - Sex all the pupae in each family. Record the date of pupa thinning and the number of males and females on the mating record.
    - Select two healthy male and two healthy female pupae from each family and place them on a lid lined with vermiculite. Each lid will be placed in a separate mating cage so to avoid inbreeding one lid should contain males from even families and females from odd families, and the other lid males from odd families and females from even families. Record the number of males and females from each family placed in the mating cage.
    - Once all pupae have been sexed, set up the mating cage. To do this, line the base with filter paper and place two 37ml pots containing cotton wool soaked with 5% honey water in each cage. Place the pupae in the cage and seal the lid with

masking tape. From the hole in the top of the lid, suspend two nappy liners. Line the hole with tissue paper and seal.

- Set up two mating cages per generation for every culture

Daily after setting up the cage:

- Check daily for adult emergence. Remove the first batch of eggs on the second day of laying.
- Replace honey water daily.

REARING PROTOCOLS FOR *H. ARMIGERA*

Authors

Chris Jones and Kenneth Wilson

Importation of new strains

The CE room must be switched on prior to arrival of insects to ensure the room is at correct temperature (26°C ±2°C)

The relevant authority should be notified prior to shipment of insects. Insects arriving from a non-EU country should pass through the AVHLA (see end for address). Those from the EU do not need to be inspected by a port authority and can be sent directly to Rothamsted. Prior to arrival Liz Isger must also be notified.

Set-up of new strains

If the insects are sent as pupae place into a clean cage with a fresh honey source and tissue attached to the side of the cage for emerging adults to rest. Mark the site of origin, name, project code and date on the cage in washable marker.

Pupae should not be transported if the journey time is more than a couple of days. This will severely hinder the development and emergence of the adults. In this case eggs maybe sent. Eggs and emerging larvae should be set-up in diet pots according to the number of insects sent.

Number of insects to rear per generation

The health and vigour of each new population will vary according to disease state, mode of transport etc. During the first generation, as many insects as feasibly possible should be reared through to adulthood. If the population looks healthy then the target per generation should be a minimum of 200 insects. Given a 50% mortality rate then this would provide approximately 100 adults and if a 50:50 sex ratio (very unlikely), 50 pairings. Of course, other factors will probably reduce the number of successful pairings further (biased sex ratio). An approximation of the number of larvae to thin from each pairing is given in Table 1.

NUMBER OF LARVAE TO THIN PER GENERATION	*No. of pairings with eggs hatched*			
Target No. of larvae for next Gen	5	10	15	20
200	40	20	13.333	10
250	50	25	16.667	12.5
300	60	30	20	15
400	80	40	26.667	20

NB: Culturing is best carried out in the order outlined below to avoid spreading any mould

Thinning:

Equipment: soft forceps, polypot lids with holes, diet pots, seed trays, blue roll, thin paint brush

Prior preparation: wash all handling equipment (e.g. forceps) with sodium hypochlorite to ensure that no mould or infection is transferred between strains.

Method:

- Select the pots that need thinning from the tray of eggs.
- Annotate the holed lids for all the pots. The pots are labelled with their family code and a lower-case letter for each individual larva e.g. AH110a, AH110b etc.
- Thin out the larvae from each pot using a thin paint brush (one insect per diet pot; careful not to damage the insect) and place in fresh diet. Place a piece of blue roll in between the lid and pot (make sure only one sheet of blue roll is inserted as larvae can get in between multiple pieces and become trapped). Place the pots in a seed tray in order on generation sheet. The seed tray should be wiped clean or washed before use to prevent transfer of mould. NB. If the total number of pots to be thinned from is less than 10, 10 larvae should be thinned from each pot. If the number of pots is greater than 10 only 5 larvae need thinning from each pot.
- The tray should be labelled with the letter L and the date.
- Annotate the correct generation sheet.

Egg collecting:

Equipment: metal spatula, scissors, polypot lids with holes, diet pots

Method:

- Select the tubs containing eggs (remember to check under the shelter as well as the tissue paper).
- Take one tub at a time and write the moth pair number (family code) and date on a polypot lid. Then remove the tissue or paper with the eggs from the chamber (don't let any moths out!). Cut the section containing the eggs out and place inside the lid with a pot of diet.
- Place pots with eggs in the correct egg tray. Repeat this for all the tubs containing eggs.
- Newly emerging female moths may lay eggs without prior mating from which no larvae will hatch. Therefore, fresh tissue should be replaced in each pot – so long as there are eggs – for either a) eggs from that pair have hatched or b) 7 days after the initial pairing after which it is unlikely that the pair will produce eggs.
- Once eggs have hatched kill the old moths in the chambers using a spatula or freezing in quarantine freezer. Ensure that they don't escape and then empty out the tubs. The lids, pots and universal lids should be kept for washing.
- Annotate the generation sheet.

NB: escaped moths must be either caught or killed. On no account should they be let out of the room

Pairing moths:

Equipment: tubs, lids, universal lids, cotton wool, filter paper (12.5cm), tissues, sucrose solution (5% sucrose)

Method:

- Collect all newly emerged moths and divide into males and females (females on the left, males on the right)
- See how many moths are in the tray of spare moths and divide any viable ones up into males and females and put with the others

- As many pairs as possible should be made up on any one day. However, if the number exceeds 20 and that generation is doing well no more than 15-20 pairs need to be set up (THEREFORE WOULD NEED 10 LARVAE PER PAIRING).
- Lay out the number of tubs required and add a shelter to each one (shelters are a piece of filter paper cut into quarters and then each quarter is folded in half to make a shelter)
- Lay out the number of universal lids required (one per tub). Fill each lid with a ball of cotton wool and saturate with sucrose solution. Add one to each tub
- Select the number of tub lids required. Each lid needs to be labelled with the moth pair number, the date and the family number of the male and female moths being used to make the pair
- The moth pair number is the next free number on the generation sheet
- The family number of the individual moths is written on their lids, this is written on the lid after the pair has been made (see below)
- The above information should be written in the following format:

  AH110 ♀ AG115c

  05/07/06 ♂ AG108f

- Enough tissue paper for all the tubs should then be prepared. Tissue paper for egg laying consists of a piece of 2 ply tissue cut in half and then divided into 1 ply pieces
- The moths can then be paired up. Sibling moths should never be put together (i.e. ones with the same family number e.g. AH110 with AH110) and only moths from the same generation should be paired.
- The female moth should be added to the tub first as they tend to be less flighty than the males.
- Once both moths have been added a piece of tissue paper is placed under the lid and the tub is then sealed.
- The family number of the male and female moths can then be added to the lid as shown in the layout above.
- Annotate the generation sheet

NB. The aim is to make 200 pairs per generation. At the end of a generation the total number of pairs made should be recorded on the sheet taped to the back of the door in CE room 5.

NB. The small lids from the polypots should be saved from the adults used to set up pairs. They are kept in a box in the CE room before being washed up and re-used (see washing up section).

Pupae collection:

Equipment: metal spatula, small plastic bag

Method:

- Go through all the larvae seed trays and collected any that have pupated. If any dead ones are found throw them away in their pot.
- For each pupa clean out the diet thoroughly and put the pupa back in the polypot. Old diet should be scraped into the plastic bag.
- Place clean pupae into a new seed tray labelled with the letter P and the date. This tray is then put on the shelf above the larvae trays.
- Pots containing mould should be cleaned out after the ones without any mould to reduce any transference. If mould is found in pots containing live larvae, they should be transferred to fresh diet pots.
- Seal the plastic bag containing the old diet when you have finished. This should be thrown away after a few days' use.

Cleaning up and waste disposal:

All equipment should be tidied away to its correct place. Equipment used to directly handle reared insects should be bleach-washed and

The work surface should be cleaned with soapy water followed by alcohol.

Waste should be placed in an autoclave bag. This should be autoclaved as soon as it is approximately half full and not left lying around.

The dirty pots and lids go next to the washing up tubs in B29 and should be washed within a few days.

DNA EXTRACTION FROM INSECTS:

Author:

Annotated by Aislinn Pearson based on the standard operating procedures described in the links below

Kit Used: Qiagen DNEasy Blood and Tissue Kit

Protocol:

Adapted from https://www.qiagen.com/gb/resources/resourcedetail?id=cabd47a4-cb5a-4327-b10d-d90b8542421e&lang=en

Using Micro-centrifuge tubes

1. Place whole insects in a 1.5 ml micro centrifuge tube.
2. Add 540 µl PBS and homogenise the sample using the electric homogenizer. I fusing the bead beater, add PBS after homogenising the sample.

NB: for homogenisation using liquid nitrogen, see relevant parts of protocol in link above

3. Centrifuge for 10 secs on maximum speed and collect 180ul of supernatant in autoclaved Eppendorf's.
4. Add 20 µl proteinase K and 200 µl Buffer AL (without added ethanol) to the supernatant.
5. Mix thoroughly by vortexing
6. Incubate at 56°C for 10 min.
7. Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing.
8. Pipet the mixture (600 µl) from step 4 (including any precipitate) into the DNEasy Mini spin column placed in a 2 ml collection tube (provided).
9. Centrifuge at ≥6000 x g (8000 rpm) for 1 min.
10. Discard flow-through and collection tube.
11. Place the DNEasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1
12. Centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.
13. Place the DNEasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2
14. Centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNEasy membrane. Discard flow-through and collection tube.
15. Following the centrifugation step, remove the DNEasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in

carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

16. Place the DNEasy Mini spin column in a clean 1.5 ml or 2 ml micro centrifuge tube (not provided), and pipet 60 µl Buffer AE directly onto the DNEasy membrane.
17. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x g (8000 rpm) to elute.
18. Store at -20˚C

DNA EXTRACTION FROM VIRUS OCCLUSION BODIES:

Author:

Annotated by Aislinn Pearson based on the standard operating procedures described in the links below

Kit Used: Qiagen DNEasy Blood and Tissue Kit

Protocol:

Adapted from https://www.qiagen.com/gb/resources/resourcedetail?id=cabd47a4-cb5a-4327-b10d-d90b8542421e&lang=en

1. Place 180µl of purified virus (concentration: c. 1 x 105 OBs/µl) in a micro centrifuge tube.
2. Add 20 µl proteinase K and 200 µl Buffer AL (without added ethanol) to the supernatant.
3. Mix thoroughly by vortexing.
4. Incubate at 56°C for 10 min.
5. Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing.
6. Pipet the mixture (600 µl) from step 4 (including any precipitate) into the DNEasy Mini spin column placed in a 2 ml collection tube (provided).
7. Centrifuge at ≥6000 x g (8000 rpm) for 1 min.
8. Discard flow-through and collection tube.
9. Place the DNEasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1
10. Centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.
11. Place the DNEasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2
12. Centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNEasy membrane. Discard flow-through and collection tube.
13. Place the DNEasy Mini spin column in a clean 1.5 ml or 2 ml micro centrifuge tube (not provided), and pipet 60 µl Buffer AE directly onto the DNEasy membrane.
14. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x g (8000 rpm) to elute.
15. Store at -20˚C

QPCR PROTOCOL:

Author:

Developed by Aislinn Pearson in conjunction with Robert Graham and Chris Jones

Kit Used: Qiagen DNEasy Blood and Tissue Kit

Protocol:

1. Use a Nanodrop to check the quality of the samples
2. Quantify the amount of DNA using the Qubit Florometer
3. To create the standard curve, use the DNA from the purified virus, calculate the copy number per µl based on the Quibit readings and using a copy calculator: http://cels.uri.edu/gsc/cndna.html (number of bp = 132,000)
4. Prepare a 10 x series dilution from the virus DNA stock for the standard curve. Values should range from 1 x 10^7 copies per µl to 1 x 10^1 copies per µl.

qPCR reagents:

- Forward primer: CTTCCCTATCGTGAACGACCAA
- Reverse primer: TGGGTCTCATGTTGATCACTAGGA
- TaqMan probe: ACACGTCCATAATTTC
- Reagents: TaqMan Fast Advanced Master Mix

Reaction conditions (as per TaqMan manufacturer instructions):

- Incubation hold: 50°C for 2 minutes
- Polymerase activation hold: 95°C for 20 seconds
- PCR cycles (x40): Denature: 95°C for 3 seconds, anneal/ extend: 60°C for 30 seconds

Experimental Design:

- Run standards, samples and non-template control in triplicate. Non-template control: RNAse-free water. Ensure samples are randomly allocated to qPCR runs and standard curve is from the same DNA extraction.

Running the qPCR:

- Defrost samples and reagents
- Vortex the probe, primers and TaqMan master mix

- Make the master mix by combining the probe, primer and Taqman Mastermix in an autoclaved Eppendorf. Calculate the total quantity required using the below quantities for each well. Calculations are based on the number of wells required plus 10%. The quantity of reagent required for each well is:

Assay mix (probe and primers)	1 µl
TaqMan	10 µl
RNAse-free water	4 µl
Total volume of master mix per well:	**15 µl**
DNA sample	*5 µl*
Total reaction volume for each well:	**20 µl**

- Vortex master mix thoroughly
- Vortex individual samples
- Pipette into well plates as appropriate
- Transfer to qPCR machine and run as specified

Once the run has completed:

- Check the quality of the standard curve. Ideal parameters are:

  R2: 0.99 or above

  Slope: -3.6 ≥ slope ≥ -3.3

  Efficiency: 90 – 110 %
- Calculate copy number of unknown samples using the RotorGene software
- Calculate the SfMNPV copy number per ug DNA: ((average copy number/5)/ quantity of DNA measured using the Qubit)*1000

APPENDIX 3: PARAMETER ESTIMATES FOR CHAPTER 2

DIFFERENCES BETWEEN FLIGHT MILL DESIGNS, *SPODOPTERA EXEMPTA*

All models are linear models (random terms instigated did not explain a significant proportion of variation)

Response	t	df	p	Mean ± se (1g)	Mean ± se (3g)
Total Distance Flown (m) (square-root scale)	4.02	64.11	<0.001 ***	102.04 ± 7.329	56.47 ± 8.28
Average Flight Duration (s) (log10 scale)	4.15	53.08	<0.001 ***	3.14 ± 0.10	2.41 ± 0.14
Maximum Flight Speed (m/s)	6.1	68.28	<0.001 ***	1.51 ± 0.08	0.87 ± 0.07
Number of Flights (log10 scale)	-3.81	59	<0.001 ***	1.07 ± 0.06	1.44 ± 0.07

DIFFERENCES BETWEEN GEOGRAPHIC POPULATIONS, *HELICOVERPA ARMIGERA*

All models are linear models (random terms instigated did not explain a significant proportion of variation)

Response	Strain	Mean (± se)	F	df	p
Total Distance Flown (m) (square-root scale)	Anyang	52.77 (± 11.23)	4.09	3, 39	0.009 **
	Dafeng	107.57 (± 12.18)			
	Jingzhou	72.3621 (± 16.08)			
	Qiuxian	70.04 (± 11.63)			
Average Flight Duration (s) (log10 scale)	Anyang	2.02 (±0.15)	3.86	3, 39	0.01 *
	Dafeng	2.86 (± 0.19)			
	Jingzhou	2.52 (± 0.34)			
	Qiuxian	2.42 (± 0.16)			
Maximum Flight Speed (m/s)	Anyang	1.29 (± 0.13)	1.59	3, 39	0.20 ns
	Dafeng	1.64 (± 0.53)			
	Jingzhou	1.70 (± 0.64)			
	Qiuxian	1.60 (± 0.66)			
Number of Flights (log10 scale)	Anyang	1.68 (± 0.11)	3.00	3, 39	0.04 *
	Dafeng	1.25 (± 0.10)			
	Jingzhou	1.37 (± 0.18)			
	Qiuxian	1.35 (± 0.10)			

FLIGHT MODELS FOR AVERAGED FLIGHT VARIABLES

SPODOPTERA EXEMPTA

All models are linear models (random terms instigated did not explain a significant proportion of variation)

Response	Predictor (second flight variable)	Coefficient	Estimate (± se)	F	p	df	Adj. R^2
Total Distance Flown (m) (square-root scale)	Number of Flights (log10 scale) †	Intercept	138.72 (± 16.50)				
		Weight of Flight Mill Arm (3g)	-32.96 (± 12.45)	16.12	<0.001 ***	2, 69	22.14%
		Number of Flights (log10 scale)	-34.34 (± 13.95)	6.06	0.016 *		
Total Distance Flown (m) (square-root scale)	Number of Flights (log10 scale) ††	Intercept	162 (± 21.25)				
		Number of Flights (log10 scale)	-62.33 (± 16.47)	14.32	< 0.001 ***	1, 66	16.58%
Average Flight Duration (s) (log10 scale)	Number of Flights (log10 scale) †	Intercept	4.47 (± 0.25)				
		Weight of Flight Mill Arm (3g)	-1.32 (± 0.49)	26.42	<0.001 ***		
		Number of Flights (log10 scale)	-1.24 (± 0.22)	31.39	<0.001 ***	3, 68	45.48%
		Interaction (3g)	0.73 (± 0.36)	4.13	0.046 *		
Average Flight Duration (s) (log10 scale)	Number of Flights (log10 scale) ††	Intercept	4.53 (± 0.25)				
		Number of Flights (log10 scale)	-1.35 (± 0.19)	50.38	<0.001 ***	1, 66	42.43%
Average Flight Duration (s) (log10 scale)	Average Flight Speed (m/s)	Intercept	2.05 (± 0.19)				
		Weight of Flight Mill Arm (3g)	-0.72 (± 0.28)	0.03	0.85 ns		
		Average Flight Speed (m/s)	2.50 (± 0.39)	103.37	<0.001 ***	3, 68	60.54%
		Interaction (3g)	2.97 (± 1.02)	8.54	0.005 **		

Number of Flights (log10 scale) †	Average Flight Speed (m/s)	Intercept	1.63 (± 0.09)			1, 70	29.72%
		Average Flight Speed (m/s)	-1.21 (± 0.22)	31.02	<0.001 ***		
Number of Flights (log10 scale) ††	Average Flight Speed (m/s)	Intercept	1.35 (± 0.11)			2, 65	34.97%
		Weight of Flight Mill Arm (3g)	0.25 (± 0.09)	8.09	0.005 **		
		Average Flight Speed (m/s)	-0.62 (± 0.23)	29.95	<0.001 ***		

† *Includes insects which engaged <3 and > 176 flights (<0.5 and >2.25 on the log10 scale)*
†† *Excludes insects which engaged <3 and > 176 flights (<0.5 and >2.25 on the log10 scale)*

HELICOVERPA ARMIGERA

All models are linear models (random terms instigated did not explain a significant proportion of variation)

Response	Predictor (second flight variable)	Coefficient	Estimate (± se)	F	p	df	Adj. R2
Total Distance Flown (m) (square-root scale)	Number of Flights (log10 scale)	Intercept	157.9 (± 15.80)			1, 71	27.64%
		Number of Flights (log10 scale)	-57.09 (± 10.77)	28.11	<0.001 ***		
Average Flight Duration (s) (log10 scale)	Number of Flights (log10 scale)	Intercept	4.21 (± 0.18)			1, 71	59.05%
		Number of Flights (log10 scale)	-1.27 (± 0.12)	104.81	<0.001 ***		
	Average Flight Speed (m/s)	Intercept	1.24 (± 0.15)			1, 71	56.52%
		Average Flight Speed (m/s)	3.58 (± 0.37)	94.59	<0.001***		
Number of Flights (log10 scale)	Average Flight Speed (m/s)	Intercept	1.89 (± 0.11)			1,71	26.77%
		Average Flight Speed (m/s)	-1.52 (± 0.29)	27.32	<0.001***		

SPODOPTERA FRUGIPERDA

Linear models (random terms instigated did not explain a significant proportion of variation)

Response	Predictor (second flight variable)	Coefficient	Estimate (± se)	F	p	df	Adj. R2
Total Distance Flown (m) (square-root scale)	Number of Flights (log10 scale)	Intercept	162.93 (± 16.59)				
		Number of Flights (log10 scale)	-58.10 (± 14.09)	3.51	0.06 .	3, 95	28.22%
		Sex (Males)	-110.50 (± 24.50)	30.57	<0.001 ***		
		Interaction (Males)	62.85 (± 23.03)	7.45	0.008 **		
Average Flight Duration (s) (log10 scale)	Number of Flights (log10 scale)	Intercept	4.47 (± 0.21)				
		Number of Flights (log10 scale)	-1.36 (± 0.18)	48.93	<0.001 ***	3, 95	44.31%
		Sex (Males)	-1.19 (± 0.31)	27.53	<0.001 ***		
		Interaction (Males)	0.62 (± 0.29)	4.51	0.04 *		
	Average Flight Speed (m/s)	Intercept	2.02 (± 0.15)				
		Average Flight Speed (m/s)	1.51 (± 0.20)	57.58	<0.001 ***	2,96	39.97%
		Sex (Males)	-0.34 (± 0.11)	9.66	0.002 **		

Linear mixed models (random terms instigated did explain a significant proportion of variation)

Response	Predictor (second flight variable)	Coefficient	Estimate (± se)	t	p	n	Estimated R2 (model)	Estimated R2 (random effects)
Number of Flights (log10 scale)	Average Flight Speed (m/s)	Intercept	1.54 (± 0.10)			99	25.87%	Date Flown: 10.59%
		Average Flight Speed (m/s)	-0.72 (± 0.12)	-6.25	<0.001 ***			
		Sex (Males)	-0.17 (± 0.07)	-2.46	0.02 *			

FLIGHT MODELS FOR EVERY FLIGHT UNDERTAKEN BY EVERY INDIVIDUAL

SPODOPTERA EXEMPTA

Response	Predictor (second flight variable)	Coefficient	Estimate (± se)	t	F	p	n (no. of groups)	Estimated R2 (model)	Estimated R2 (random effects)
Flight duration (s) (log10 scale)	Maximum Flight Speed (m/s)	Intercept	1.85 (± 0.07)	24.99			1,273 (71)	55.05%	Individual: 19.08%
		Maximum Flight Speed (m/s)	1.05 (± 0.05)	20.51	578.33	<0.001 ***			
		Arm weight (3g)	-0.04 (± 0.11)	-0.42	0.18	0.67 ns			
		Interaction (3g)	0.22 (± 0.10)	2.24	2.24	0.03 *			
	Average Flight Speed (m/s)	Intercept	2.33 (± 0.09)	26.28			1,273 (71)	39.15%	Individual: 34.07%
		Average Flight Speed (m/s)	1.16 (± 0.12)	9.99	187.85	<0.001 ***			
		Arm weight (3g)	-0.39 (± 0.13)	-2.98	8.86	0.004 **			
		Interaction (3g)	0.59 (± 0.21)	2.77	7.75	0.006**			

HELICOVERPA ARMIGERA

Response	Predictor (second flight variable)	Coefficient	Estimate (± se)	t	F	p	n (no. of groups)	Estimated R2 (model)	Estimated R2 (random effects)
Flight duration (s) (log10 scale)	Maximum Flight Speed (m/s)	Intercept	1.68 (± 0.08)	20.55			1,476 (67)	65.10%	Individual: 17.62%
		Maximum Flight Speed (m/s)	0.54 (± 0.04)	12.28	1118.64	< 0.001 ***			
		Strain (Dafeng)	-0.16 (± 0.11)	-1.44					
		Strain (Jingzhou)	-0.16 (± 0.14)	-1.09	0.80	0.50 ns			
		Strain (Qiuxian)	-0.08 (± 0.11)	-0.747					
		Interaction (Dafeng)	0.51 (± 0.06)	8.66					
		Interaction (Jingzhou)	0.32 (± 0.07)	4.07	25.35	<0.001 ***			
		Interaction (Qiuxian)	0.33 (± 0.06)	5.29					
Flight duration (s) (log10 scale)	Average Flight Speed (m/s)	Intercept	1.73 (± 0.12)	14.67			1,476 (67)	39.25%	Individual: 40.52%
		Average Flight Speed (m/s)	0.91 (± 0.09)	10.13	740.02	< 0.001 ***			
		Strain (Dafeng)	0.03 (± 0.16)	0.18					
		Strain (Jingzhou)	-0.01 (±0.21)	-0.07	0.07	0.98 ns			
		Strain (Qiuxian)	-0.04 (± 0.16)	-0.26					
		Interaction (Dafeng)	0.85 (± 0.13)	6.70					
		Interaction (Jingzhou)	0.44 (± 0.16)	2.74	15.49	<0.001 ***			
		Interaction (Qiuxian)	0.56 (± 0.13)	4.47					

SPODOPTERA FRUGIPERDA

Response		Coefficient	Estimate (± se)	t	F	p	n (n groups)	Estimated R2 (model)	*Estimated R^2 (random effects)*
Flight duration (s) (log10 scale)	Maximum Flight Speed (m/s)	Intercept	1.42 (± 0.05)	26.83			2,267 (110)	44.07%	Individual: 20.34
		Maximum Flight Speed (m/s)	1.09 (± 0.03)	31.65	1192.31	<0.001 ***			
		Sex (Males)	0.21 (± 0.08)	2.61	6.80	0.009 **			
		Interaction (Males)	-0.34 (± 0.05)	-6.41	41.12	<0.001 ***			
	Average Flight Speed (m/s)	Intercept	1.67 (± 0.06)	26.71			2,267 (110)	30.52%	Individual: 24.51
		Average Flight Speed (m/s)	1.43 (± 0.07)	21.39	472.54	<0.001 ***			
		Sex (Males)	0.24 (± 0.09)	2.53	6.38	0.01 *			
		Interaction (Males)	-0.60 (± 0.10)	-5.71	33.30	<0.001 ***			

APPENDIX 4: PARAMETER ESTIMATES FOR CHAPTER 3

MORTALITY

General Linear Models (GLM)

Response	Coefficient	Estimate (± se)	Z	p	Deviance
logit (proportion of insects surviving NPV infection)	Intercept	8.74 (± 2.03)	4.3		
	Pathogen Challenge (OBs per µl) (log10 scale)	-1.51 (± 0.44)	-3.44	<0.001 ***	26.47
logit (proportion of insects dying in the pupal phase)	No significant terms	na	na	> 0.05 ns	na
logit (proportion of larvae failing to pupate)	No significant terms	na	na	> 0.05 ns	na

FLIGHT BEHAVIOUR MODELS

Linear Models (LM)

Response	Coefficient	Estimate (± se)	F	p	df	Adj. R2
PCA scores for first component	Intercept	-0.72 (± 0.81)			3, 95	11.77%
	Pathogen Challenge (OBs per µl) (log10 scale)	-0.06 (± 0.26)	2.7	0.103 ns		
	Sex (Males)	-0.32 (± 1.24)	8.55	0.004 **		
	Interaction (Males)	0.88 (± 0.40)	4.82	0.030 *		
PCA scores for second component	Intercept	-0.28 (± 0.17)			1, 97	5.02%
	Sex (Males)	0.63 (± 0.26)	6.18	0.015 *		

Total Distance Flown (m) (square-root scale)	Intercept	86.84 (± 10.94)			3, 112	28.37%
	Pathogen Challenge (OBs per µl) (log10 scale)	3.92 (± 3.58)	1.21	0.273 ns		
	Sex (Males)	-23.09 (± 16.06)	42.08	<0.001 ***		
	Interaction (Males)	-11.98 (± 5.17)	5.38	0.022 *		
Average Flight Duration (s) (log10 scale)	Intercept	2.86 (± 0.16)			3, 95	11.56%
	Pathogen Challenge (OBs per µl) (log10 scale)	0.04 (± 0.05)	1.52	0.220 ns		
	Sex (Males)	0.17 (± 0.25)	7.55	0.007 **		
	Interaction (Males)	-0.21 (± 0.08)	6.74	0.010 *		
Maximum Flight Speed (m/s)	No significant terms	na	na	> 0.05 ns	na	na

Linear Mixed Effects Models (LME)

Response	Coefficient	Estimate (± se)	t	F	p	n	Estimated R^2 (model)	Estimated R^2 (random effects)
Number of Flights (log10 scale)	Intercept	1.2 (± 0.14)	8.35			99	10.11%	Date Flown: 7.68% Sibling Group: 8.25%
	Pathogen Challenge (OBs per µl) (log10 scale)	-0.15 (± 0.11)	-1.4	2.43	0.1222 ns			
	Pathogen Challenge2 (OBs per µl) (log10 scale)	0.02 (± 0.02)	1.2	2.24	0.138 ns			
	Sex (Males)	-0.80 (± 0.21)	-3.79	13.74	<0.001 ***			
	Interaction (Males)	0.62 (± 0.18)	3.45	11.28	0.001 **			
	Interaction2 (Males)	0.10 (± 0.03)	-3.18	9.87	0.002 **			

DEVELOPMENT MODELS

Linear Mixed Effects Models (LME)

Response	Coefficient	Estimate (± se)	t	F	p	*n*	*Estimated R^2 (model)*	*Estimated R^2 (random effects)*
Total Development Time (TDT) (days)	Intercept	18.13 (± 0.66)	27.63			210	39.43%	Diet Batch: 5.36% Sibling Group: 6.09%
	Pathogen Challenge (OBs per µl) (log10 scale)	-0.15 (± 0.06)	-2.79	4.08	0.045*			
	Sex (Males)	0.62 (± 0.24)	2.52	6.39	0.012 *			
	Pupal Weight (mg)	0.01 (± 0.002)	4.46	19.95	<0.001 ***			
	Larval Weight at Infection (mg)	-0.09 (± 0.009)	-9.42	88.77	<0.001 ***			
	Sex: Pathogen Challenge Interaction (Males)	0.16 (± 0.08)	2.03	4.11	0.044 *			
Larval Development Time (LDT) (days)	Intercept	11.36 (± 0.23)	49.77			210	26.40%	Diet Batch: 11.33%
	Larval Weight at Infection (mg)	-0.07 (± 0.01)	-8.31	68.96	<0.001 ***			
Pupal Development Time (PDT) (days)	Intercept	8.81 (± 010)	84.45			210	51.15%	Sibling Group: 5.06%
	Pathogen Challenge (OBs per µl) (log10 scale)	-0.08 (± 0.02)	-3.46	11.98	<0.001 ***			
	Sex (Males)	1.11 (± 0.07)	15.43	283.12	<0.001 ***			

PUPAL AND ADULT WEIGHT MODELS

Linear Mixed Effects Models (LME)

Response	Coefficient	Estimate (± se)	t	F	p	n	Estimated R^2 (model)	Estimated R^2 (random effects)
Pupal Weight (mg)	Intercept	216.67 (± 8.08)	26.82			210	4.56%	Sibling Group: 4.06% Diet Batch: 11.12%
	Larval Weight at Infection (mg)	0.069 (± 0.25)	2.74	7.53	0.007 **			
Proportional Weight Loss During the Pupal Phase (mg)	Intercept	90.66 (± 5.52)	16.44			153	7.41%	Diet Batch: 8.83%
	Sex (Males)	10.91 (± 2.78)	3.92	15.38	0.001 ***			
	Larval Weight at Infection (mg)	0.46 (± 0.19)	2.42	5.84	0.012 **			
Proportional Weight Gain During Feeding (mg) (square root scale)	Intercept	0.21 (± 0.01)	14.88			126	2.24%	Date Flown: 9.66%
	Sex (Males)	-0.03 (± 0.01)	-2.15	4.6	0.034			
Proportional Weight Loss After Flight (mg)	Intercept	0.12 (± 0.01)	15.8			135	10.73%	Date Flown: 10.02%
	Pathogen Challenge (OBs per µl) (log10 scale)	0.006 (± 0.002)	2.62	0.67	0.41 ns			
	Sex (Males)	0.01 (± 0.01)	1.16	1.35	0.25 ns			
	Interaction (Males)	0.009 (± 0.003)	-2.92	8.55	0.004 **			

Linear models (LM)

Response	Coefficient	Estimate (± se)	F	p	df	Adj. R^2
Adult Emergence Weight (mg)	Intercept	178.18 (± 3.32)			2, 157	10.33%
	Sex (Males)	-9.29 (± 2.36)	15.43	<0.001 ***		
	Larval Weight at Infection (mg)	0.37 (± 0.16)	5.17	0.024 *		
Post Flight Weight (mg)	Intercept	156.10 (± 3.38)			2, 136	7.01%
	Sex (Males)	-6.18 (± 2.43)	6.46	0.012 *		

	Larval Weight at Infection (mg)	0.42 (± 0.17)	6.39	0.013 *

WING LENGTH MODELS

RELATIONSHIP BETWEEN WING LENGTH AND FLIGHT VARIABLES

Linear Models (LM)

Response	Coefficient	Estimate (± se)	F	p	df	Adj. R^2
Total Distance Flown (m) (square root scale)	Intercept	-214.21 (± 123.84)			1, 84	4.77%
	Average Wing Length (mm)	18.73 (± 8.17)	5.25	0.02 *		
Average Flight Duration (s) (log10 scale)	Intercept	0.66 (± 1.67)			1, 72	0.93%
	Average Wing Length (mm)	0.14 (± 0.11)	1.68	0.2 ns		

Linear Mixed Effects Models (LME)

Response	Coefficient	Estimate (± se)	t	p	n	Estimated R2 (model)	Estimated R2 (random effects)
Number of Flights (log10 scale)	Intercept	-0.83 (± 1.16)			86 (2)	14.75%	Diet batch: 7.75%
	Average Wing Length (mm)	0.11 (± 0.08)	1.47	0.15 ns			
Maximum Flight Speed (m/s)	Intercept	-0.53 (± 1.17)			74 (2)	19.06%	Mill Set: 13.89%
	Average Wing Length (mm)	0.14 (± 0.08)	1.89	0.06 ns			

Relationship with physiological and development terms

Linear Models (LM)

Response	Coefficient	Estimate (± se)	F	p	df	Adj. R2
Wing Length (mm)	Intercept	12.53 (± 0.55)			1, 84	20.32%
	Pupal Weight (mg)	0.011 (± 0.002)	22.67	<0.001 ***		
Wing Length (mm)	Intercept	17.23 (± 1.14)			1, 84	4.70%
	Development Time (days)	-0.13 (± 0.06)	5.19	0.030 *		
Wing Length (mm)	Intercept	15.40 (± 0.51)			1, 84	13.78%
	Sex (males)	-0.51 (± 13.36)	14.59	<0.001 ***		

Relationship with virus load

Linear Models (LM)

Response	Coefficient	Estimate (± se)	F	p	df	Adj. R2
Wing Length (mm)	Intercept	12.80 (± 0.50)			2, 83	34.44%
	Sex (males)	0.011 (± 0.002)	27.56	<0.001 ***		
	Pupal Weight (mg)	-0.51 (± 0.12)	19.09	<0.001 ***		

VIRUS LOAD MODELS

Linear Mixed Effects Models (LME) – Not included in reported results

Response	Flight variable of interest	Coefficient	Estimate (± se)	t	F	p	n	Estimated R^2 (model)	Estimated R^2 (random effects)
Viral load (DNA copies per ng host DNA) (log10 scale) †	Total Distance Flown (m)†	Intercept	3.63 (± 0.25)	14.53			84 (6)	-1.78%	Processing batch: 72.30%
		Total Distance Flown (m)	0.11 (± 0.13)	0.81	2.43	0.123 ns			
		Virus Challenge (OBs per µl) (log10 scale)	0.00 (± 0.04)	0.06	0.8	0.373 ns			
		Sex	0.15 (± 0.19)	0.8	0.64	0.428 ns			
		Total Distance: Sex (Males)	-0.71 (± 0.31)	-2.27	5.14	0.026 *			
		Total Distance: Virus Challenge	-0.02 (± 0.04)	-0.69	2.72	0.103 ns			
		Virus Challenge: Sex	-0.06 (± 0.06)	-0.99	0.99	0.324 ns			
		Total Distance: Virus Challenge: Sex	0.23 (± 0.10)	2.24	5	0.028 *			
	Total Distance Flown (m)††	No significant terms	na	na	na	<0.05 ns	84 (6)	na	na
	Average Flight Duration (s)†††	Intercept	3.64 (± 0.20)	18.32			84 (6)	-0.02%	Processing batch: 68.70 %
		Average Flight Duration (s)	0.01 (± 0.04)	0.14	5.22	0.026 *			
		Sex (Males)	-0.002 (± 0.09)	-0.03	0.98	0.976 ns			
		Average Flight Duration: Sex	-4.11 (± 0.18)	-2.32	5.4	0.023 *			
	Average Flight Duration (s) (log10 scale) ††	No significant terms	na	na	na	<0.05 ns	84 (6)	na	na
	Average Flight Duration (s)†††	No significant terms	na	na	na	<0.05 ns	81 (6)	na	na

Viral load (DNA copies per ng host DNA) (log10 scale) [†]	Number of Flights	No significant terms	na	na	na	<0.05 ns	84 (6)	na	na
	Maximum Flight Speed (m/s)	No significant terms	na	na	na	<0.05 ns	72 (6)	na	na

[†] Includes an influential observation (Cook's distance = 0.3)
[††] Excludes influential observation
[†††] Includes single male with a flight duration over 5,000 seconds (circa 80 minutes)

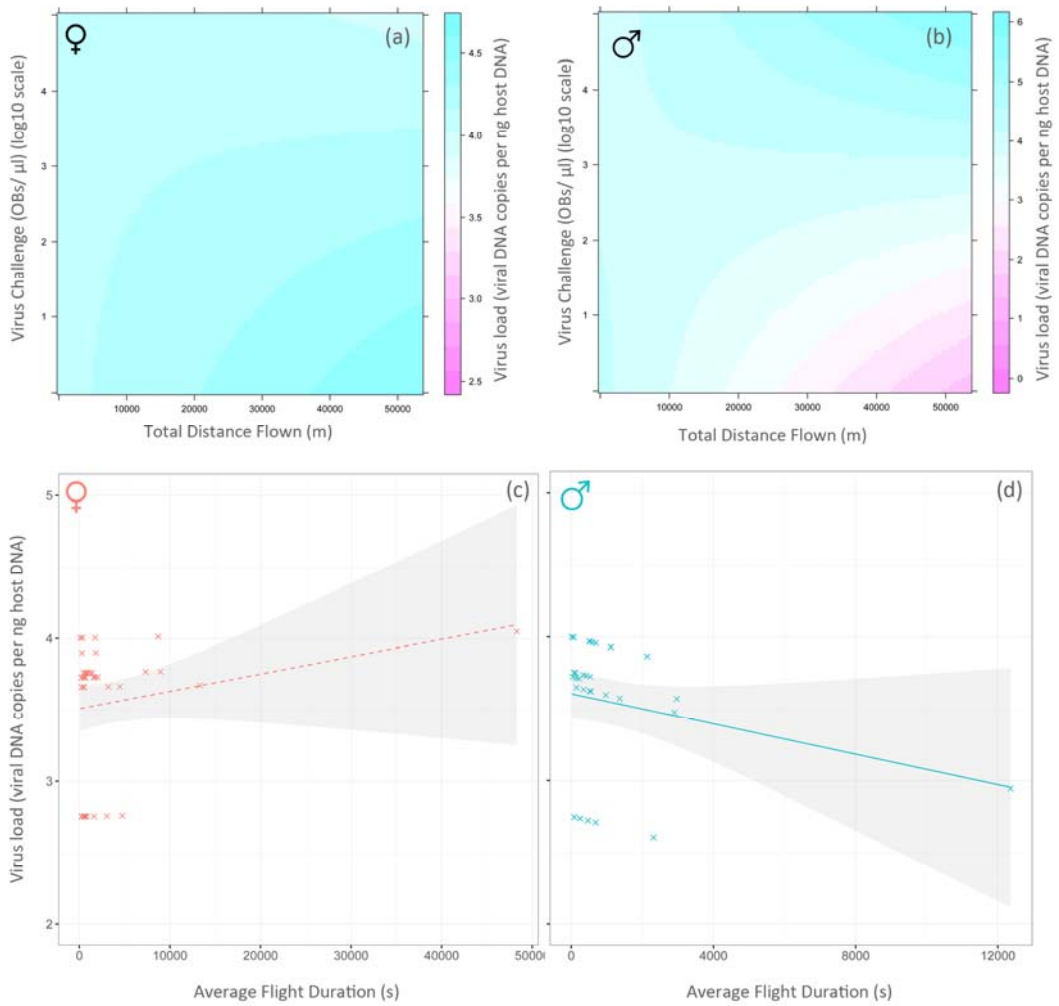

Figure 0.1: The relationship between sex, virus challenge, pathogen load and flight behaviour. Viral load was described by a three-way interaction between total distance flown, sex and pathogen challenge (top). In females (a), this resulted in increases in viral load at low levels of pathogen challenge and over large distances flown. In males (b), the opposite was true: pathogen challenge was lowest at low levels of infection and over greater distances. In males, the highest viral loads were found in insects with exposed to the highest levels of pathogen challenge which flew greater distances. Average flight duration was described by a two-way interaction between sex and average flight duration (bottom), where the relationship between the two variables was not significant for females (c) but in males (d), increases in flight duration lead to a decrease in viral load. This relationship was no significant if average flight duration was log10 transformed or if the male that flew for more than 1,200 seconds was removed from the dataset.

APPENDIX 5: PARAMETER ESTIMATES FOR CHAPTER 4

MORTALITY

FIVE HOUR FORCED-FLIGHT TRIAL

General Linear Models (GLM)

Response	Coefficient	Estimate (± se)	Z	p	Deviance
logit (proportion of insects surviving NPV infection)	Intercept	6.24 ± 0.60	10.42		
	Pathogen Challenge (OBs per µl) (log10 scale)	-1.71 ± 0.15	-11.06	<0.001 ***	11.75
logit (proportion of insects dying in the pupal phase)	No significant terms	na	na	> 0.05 ns	na
logit (proportion of larvae failing to pupate)	No significant terms	na	na	> 0.05 ns	na

MANIPULATED FLIGHT TIME TRIAL

General Linear Models (GLM)

Response	Coefficient	Estimate (± se)	Z	p	Deviance
logit (proportion of insects surviving NPV infection)	Intercept	3.58 ± 0.30	12.14		
	Pathogen Challenge (OBs per µl) (log10 scale)	-1.02 ± 0.09	-11.27	<0.001 ***	16.59
logit (proportion of insects dying in the pupal phase)	No significant terms	na	na	> 0.05 ns	na
logit (proportion of larvae failing to pupate)	No significant terms	na	na	> 0.05 ns	na

DEVELOPMENT MODELS

FIVE HOUR FORCED-FLIGHT TRIAL

Linear Mixed Effects Models (LME)

Response	Coefficient	Estimate (± se)	t	F	p	n	Estimated R^2 for fixed effects	Estimated R^2 for random effects (number of groups)
Total Development Time (TDT) (days)	Intercept	17.17 (± 0.54)	31.82			270	59.56%	Date Infected (9): 4.87% Sibling Group (8): 1.62% Diet Batch (2): 1.65%
	Sex (Males)	1.11 (± 0.10)	11.51	132.42	<0.001 ***			
	Pupal Weight (mg)	0.008 (± 0.002)	4.23	17.85	<0.001 ***			
	Larval Weight at Infection (mg)	-0.05 (± 0.003)	-14.12	199.29	<0.001 ***			
Larval Development Time (LDT) (days)	Intercept	11.23 (± 0.18)	63.24			276	49.00%	Date Infected (9): 2.45% Sibling Group (8): 7.44% Diet Batch (2): 1.17%
	Larval Weight (mg)	-0.046 (± 0.003)	-16.44	270.25	<0.001 ***			
Pupal Development Time (PDT) (Days)	Intercept	8.24 (± 0.11)	73.29			270	53.92%	Date Infected (9): 1.50% Sibling Group (8): 1.44%
	Pathogen Challenge (OBs per µl) (log10 scale)	-0.04 (± 0.03)	-1.37	0.07	0.79			
	Sex (Males)	0.89 (± 0.10)	8.66	74.96	<0.001 ***			
	Larval Weight at Infection (mg)	-0.006 (± 0.002)	-2.81	7.87	0.006 **			
	Sex: Pathogen Challenge Interaction (Males)	0.10 (± 0.04)	2.28	5.21	0.023 *			

MANIPULATED FLIGHT TIME TRIAL

Linear Mixed Effects Models (LME)

Response	Coefficient	Estimate (± se)	t	F	p	*n*	Estimated R^2 for fixed effects	Estimated R^2 for random effects (number of groups)
Total Development Time (TDT) (days)	Intercept	19.71 (± 0.13)	155.53			418	59.68%	Date Infected (8): 2.02%
	Sex (Males)	0.95 (± 0.07)	12.84	164.88	<0.001 ***			
	Larval Weight at Infection (mg)	-0.06 (± 0.003)	-21.14	447.02	<0.001 ***			
Larval Development Time (LDT) (days)	Intercept	11.50 (± 0.11)	103.47			418	56.46%	Sibling Group (9): 3.56%
	Larval Weight (mg)	-0.06 (± 0.003)	-21.91	480.06	<0.001 ***			
Pupal Development Time (PDT) (days)	Intercept	8.11 (± 0.05)	173.73			418	49.25%	Sibling group (9) 1.45%
	Sex (Males)	1.04 (± 0.05)	415.1	409.73	<0.001 ***			

WEIGHT MODELS

FIVE HOUR FORCED-FLIGHT TRIAL

Linear Mixed Effects Models (LME)

Response	Coefficient	Estimate (± se)	t	F	p	n	Estimated R2 (model)	Estimated R2 (random effects)
Pupal Weight (mg)	No significant terms	na	na	na	<0.05	216	na	na
Pre-Flight Weight (mg)	Intercept	145.45 (± 4.44)	32.77			211	12.92%	Date Infected (9): 2.32% Sibling Group (8): 9.94%
	Pathogen Challenge (OBs per µl) (log10 scale)	2.04 (± 1.51)	1.35	0.12	0.73 ns			
	Sex (Males)	-8.91 (± 4.70)	-1.9	3.61	0.06 .			
	Sex (Males): Pathogen Challenge Interaction	-4.78 (± 2.05)	-2.33	5.44	0.02 *			
Post-Flight Weight (mg)	Intercept	138.18 (± 4.05)	34.1			180	21.89%	Sibling Group (8): 4.12%
	Sex (Males)	-16.98 (± 2.65)	-6.41	41.12	<0.001 ***			
	Flown (No)	5.55 (± 2.64)	2.1	4.42	0.037 *			
	Larval Weight at Infection (mg)	-2.45 (± 0.09)	-2.77	7.68	0.006 **			
Proportional Weight Loss During Flight (mg)	Intercept	0.11 (± 0.009)	12.29			180	6.14%	Diet Batch (2): 6.14%
	Sex (Males)	0.017 (± 0.006)	3.15	9.92	0.002 **			
	Flown (No)	-0.036 (± 0.005)	-6.57	43.15	< 0.001 ***			

Linear models (LM)

Response	*Coefficient*	*Estimate (± se)*	*t*	*F*	*p*	*df*	*Adj. R^2*
Proportional Weight Loss (Pupae)	Intercept	0.44 (± 0.006)	84.77			1, 209	16.36%
	Sex (Males)	0.06 (± 0.009)	-6.49	42.08	<0.001***		

MANIPULATED FLIGHT TIME TRIAL

Linear Mixed Effects Models (LME)

Response	Coefficient	Estimate (± se)	t	F	p	n	Estimated R^2 (model)	Estimated R^2 (random effects)
Pupal Weight (mg)	Intercept	237.92 (± 7.06)	33.72			418	3.50%	Sibling Group (9): 22.03%
	Larval Weight at Infection (mg)	0.41 (± 0.13)	3.03	9.19	0.003 **			
	Sex (Males): Pathogen Challenge Interaction	-4.78 (± 2.05)	-2.33	5.44	0.02 *			
Pre-Flight Weight (mg)	Intercept	138.29 (± 4.82)	28.69			140	12.55%	Sibling Group (9): 15.31%
	Pathogen Challenge (OBs per µl) (log10 scale)	-2.84 (± 1.33)	-2.14	4.58	0.034 *			
	Sex (Males)	-19.36 (± 3.92)	-4.94	24.36	<0.001 ***			
Pre-Flight Weight (mg)[††]	Intercept	132.65 (± 4.30)	30.86			138	14.79%	Sibling Group (9): 22.44%
	Sex (Males)	-18.54 (± 3.23)	-5.61	31.53	< 0.001 ***			
Post-Flight Weight (mg) (all treatments, factorial flight treatment)	Intercept	121.27 (± 3.06)	36.65			140	21.25%	Date Flown: 5.14%
	Sex (Males)	-18.12 (± 3.25)	-5.57	31.02	<0.001 ***			
Post-Flight Weight (mg)[†††] (all treatments, factorial flight treatment)	Intercept	112.87 (± 4.49)	25.01			139	25.24%	Date Flown: 2.43%
	Sex (Males)	-18.03 (± 3.05)	-5.91	34.97	<0.001 ***			
	Larval Weight at Infection (mg)	2.49 (± 1.19)	2.1	4.4	0.038 *			
Post-Flight Weight (mg) (flown insects only, continuous flight treatment)	Intercept	126.89 (± 5.19)	24.44			112	28.03%	Date Flown: 10.30%
	Sex (Males)	-31.52 (± 6.34)	-4.98	24.73	<0.001 ***			
	Time Spent Flying (h)	-2.52 (± 1.33)	-1.87	0.02	0.90 ns			
	Sex: Time Spent Flying Interaction	4.78 (± 1.91)	2.51	6.3	0.014 *			

Proportional Weight Loss (Flown Adults) (mg) (flown insects only, continuous flight treatment)	Intercept	0.07 (± 0.007)	11.23			110	58.73%	Date Flown: 2.32%
	Sex (Males)	0.01 (± 0.005)	2.73	7.45	0.007 **			
	Time Spent Flying (h)	0.007 (± 0.002)	4.72	22.67	<0.001 ***			

†† Excludes two influential observations (cooks' distance <0.1), a female with a pre-flight weight of 215.1 mg and a male with a pre-flight weight of 242 mg. Both are in the control group.
††† Excludes an influential observation (cooks distance < 0.12), an non-flown female in the control treatment group with a post flight weight of 203 mg.

Linear models

Response	***Coefficient***	***Estimate (± se)***	***t***	***F***	***p***	***df***	***Adj. R^2***
Proportional Weight Loss (Pupae)	Intercept	0.47 (± 0.008)	57.6			1, 138	16.82%
	Sex (Males)	0.06 (± 0.01)	5.4	29.1	<0.001 ***		
Proportional Weight Loss (Flown Adults) (mg) (all treatments, factorial flight treatment)	Intercept	0.09 (± 0.006)	14.68			3, 135	5.70%
	Flight Treatment (3h)	0.01 (± 0.009)	1.44	3.78	0.012 *		
	Flight Treatment (5h)	0.02 (± 0.009)	2.69				
	Flight Treatment (NF)	-0.005 (± 0.01)	-0.55				

VIRUS LOAD MODELS

FIVE HOUR FORCED-FLIGHT TRIAL: INTERACTION BETWEEN SEX, FLIGHT TREATMENT AND VIRUS CHALLENGE

Linear Mixed Effects Models (LME)

Response	Coefficient	Estimate (± se)	t	F	p	n	Estimated R^2 (model)	Estimated R^2 (random effects)
Viral load (DNA copies per ng host DNA) (log10 scale)	Intercept	3.57 (± 0.32)	11.87			156	7.86%	Processing batch: 35.46%
	Flown (No)	0.18 (± 0.06)	-2.98	8.9	0.003 **			
	Sex (Females)	-0.20 (± 0.06)	-3.31	10.95	0.001 **			
	Pupal Weight (mg)	-0.002 (± 0.001)	-2.03	4.13	0.044 *			

MANIPULATED FLIGHT TIME TRIAL: INTERACTION BETWEEN SEX, FLIGHT TREATMENT AND VIRUS CHALLENGE

Linear Mixed Effects Models (LME)

Response	Coefficient	Estimate (± se)	t	χ^2	p	n	Estimated R^2 (model)	Estimated R^2 (random effects)
Viral load (DNA copies per ng host DNA) (log10 scale)	Intercept	3.68 (± 0.18)	20.51			175	3.38%	Processing batch: 60.50%
	Flight Treatment (3h)	-0.13 (± 0.08)	-1.62	22.03	< 0.001 ***			
	Flight Treatment (5h)	-0.16 (± 0.08)	-1.87					
	Flight Treatment (Frozen on emergence)	0.18 (± 0.08)	2.3					
	Flight Treatment (Not Flown)	-0.10 (± 0.09)	-1.1					
	Pathogen Challenge (OBs per µl) (log10 scale)	-0.03 (± 0.03)	-0.99	0	1.00 ns			
	Pupal Weight (mg)	-1.41 (± 0.56)	-2.52	6.62	0.010 *			
	Pathogen Challenge: Flight Treatment (3h) Interaction	0.03 (± 0.04)	0.84	9.95	0.041 *			
	Pathogen Challenge: Flight Treatment (5h) Interaction	0.08 (± 0.04)	1.93					
	Pathogen Challenge: Flight Treatment (Frozen on emergence) Interaction	-0.04 (± 0.04)	-1.03					
	Pathogen Challenge: Flight Treatment (Not Flown) Interaction	0.01 (± 0.05)	0.32					

APPENDIX 6: PARAMETER ESTIMATES FOR CHAPTER 5

LINEAR RELATIONSHIPS BETWEEN VIRUS LOAD, WEIGHT, WING LENGTH AND POPULATION DENSITY

Response	Parameter estimates	Model	Test statistics		
			F	df/ n	p
Viral load (DNA copies per ng host DNA) (log10 scale)	Weight (mg) (log10 scale)	LME	0.7	624	0.4 ns
Viral load (DNA copies per ng host DNA) (log10 scale)	Wing Length (mm)	LME	0.04	507	0.84 ns
Viral load (DNA copies per ng host DNA) (log10 scale)	Number of insects caught (log10 scale)	LM	1.14	1, 77	0.29 ns
Weight (mg) (log10 scale)	Wing length (mm)	LME	0.6	507	0.44 ns
Weight (mg) (log10 scale)	Number of insects caught (log10 scale)	LM	0.22	1, 77	0.65 ns
Wing length (mm)	Weight (mg) (log10 scale)	LM	0.25	1, 505	0.61 ns
Wing length (mm)	Number of insects caught (log10 scale)	LM	1.18	1, 60	0.28 ns
Number of insects caught (log10 scale)	Wing length (mm)	LME	1.4	62	0.24 ns
Number of insects caught (log10 scale)	Weight (mg) (log10 scale)	LME	0.03	79	0.86 ns

TEMPORAL VARIATION IN VIRUS LOAD, WEIGHT, WING LENGTH AND POPULUATION DENSITY

Response	Explanatory variable	Model	Nonlinearity test statistics		Linear model test statistics			Adj. R^2/ Estimated R^2 for fixed effects	Estimated R^2 for random effects (number of groups)
			F/ X^2	p	F	df/ n	p		
Viral load (DNA copies per ng host DNA) (log10 scale)	Month (24 level factor)	LME	44.8	0.003 **	1.98	624	0.006 **	8.32%	Processing Batch (38): 16.37%; Sample location (9): 0.21%
Weight (mg) (log10 scale)	Month (24 level factor)	LME	168.04	<0.001 ***	8.48	624	<0.001 ***	7.91%	Sample location (9): 22.82%
Wing length (mm)	Month (24 level factor)	LME	26.75	0.221 ns	1.19	507	0.27	1.72%	Processing Batch (38): 3.17%
Number of insects caught (log10 scale)	Month (24 level factor)	LM	1.5	0.11 ns	8.48	23, 55	<0.001	23.37%	-

Response	Location, Year	Model	Nonlinearity test statistics		Linear model test statistics			Adj. R2/ Estimated R2 for fixed effects	Estimated R2 for random effects (number of groups)
			F/ X^2	p	F	df/ n	p		
Viral load (DNA copies per ng host DNA) (log10 scale)	Nueces, TX. 2012	LM	1.52	0.13 ns	1.43	13, 93	0.16 ns	-	-
	Nueces, TX. 2013	LM	3.97	0.002 **	3.82	8, 44	0.002 **	30.26%	-
	Alachua, FL. 2012	LME	5.91	0.55 ns	0.6	93	0.76 ns	-	Processing Batch: 32.48%
	Alachua, FL. 2013	LM	1.46	0.19	1.31	9, 63	0.25 ns	-	-
	All sample locations, 2012	LME	7.26	0.7	0.65	354	0.78 ns	1.21%	Processing Batch (20): 14.59% Sample location (9): 2.73%
	All sample locations, 2013	LME	27.18	0.002 **	3.26	270	<0.001 ***	21.32%	Processing Batch (19): 13.71% Sample location (9): 64.96%
Wing length (mm)	Nueces, TX. 2012	LM	0.82	0.63 ns	0.84	13, 74	0.62 ns	-	-
	Nueces, TX. 2013	LM	1.18	0.34 ns	1.6	8, 34	0.16 ns	-	-
	Alachua, FL. 2012	LM	1.11	0.37 ns	1.39	8, 65	0.22 ns	-	-
	Alachua, FL. 2013	LM	0.68	0.71 ns	1.39	9, 46	0.78 ns	-	-
	All sample locations, 2012	LM	0.97	0.47 ns	1.4	11, 276	0.17 ns	-	-
	All sample locations, 2013	LM	1.58	0.12 ns	1.58	11, 207	0.11 ns	-	-

Weight (mg) (log10 scale)	Nueces, TX. 2012	LM	4.6	<0.001 ***	5.14	13, 93	< 0.001 ***	33.64%	-
	Nueces, TX. 2013	LM	11.17	< 0.001 ***	12.41	8, 44	< 0.001 ***	63.71%	-
	Alachua, FL. 2012	LM	9.95	<0.001 ***	10.27	8, 84	<0.001 ***	44.62%	-
	Alachua, FL. 2013	LM	7.02	<0.001 ***	6.47	9, 63	<0.001 ***	40.59%	-
	All sample locations, 2012	LM	4.25	<0.001 ***	4.11	11, 342	<0.001 ***	8.84%	-
	All sample locations, 2013	LM	5.65	<0.001 ***	5.44	25, 244	<0.001 ***	29.23%	-
Number of insects caught (log10 scale)	All sample locations, 2012	LM	2.43	0.03 *	2.44	11, 29	0.027 *	28.30%	-
	All sample locations, 2013	LME	20.4	0.026 *	2.53	38	0.032 *	3.56%	Sample location (9): 3.56%

Year	Response	Model	Linear model test statistics				Adj. R^2/ Estimated R2 for fixed effects	Estimated R^2 for random effects (number of groups)
			Coefficient	F	p	df/ n		
2012	Viral load (DNA copies per ng host DNA) (log10 scale)	LME	No significant terms	-	-	354	-	-
	Weight (mg) (log10 scale)	LME	No significant terms	-	-	354	-	-
2013	Viral load (DNA copies per ng host DNA) (log10 scale)	LME	Migratory Season	4.58	0.037 *	270	2.56%	Processing batch (19): 6.86 % Sampling site (9): 23.86 %
	Weight (mg) (log10 scale)	LME	Migratory Season	9.03	0.004 **	270	11.53%	Processing batch (19): 14.48 % Sampling site (9): 10.96 %

Migratory Pathway, Year	Response	Model	Nonlinearity test statistics		Linear model test statistics				Adj. R^2/ Estimated R^2 for fixed effects	Estimated R2 for random effects (number of groups)
			F/ X^2	p	Coefficient	F	p	df/ n		
West, 2012	Viral load (DNA copies per ng host DNA) (log10 scale)	LME	1.03	0.03 ns	No significant terms	-	-	124	-	-
East, 2012	Viral load (DNA copies per ng host DNA) (log10 scale)	LME	0.36	0.83 ns	No significant terms	-	-	89	-	-
West, 2013	Viral load (DNA copies per ng host DNA) (log10 scale)	LM	0.02	0.98 ns	North/ South	20.91	<0.001 ***	1, 88	18.29%	-
	Weight (mg) (log10 scale)	LM	6.1	0.003 **	North/ South	22.51	<0.001 ***	4, 85	28.70%	-
					Month (factor)	5.77	0.001 **			
	Wing length (mm)	LM	0.54	0.58 ns	North/ South	2.08	0.041 *	1, 71	4.49%	-
	Number of insects caught (log10 scale)	LM	1.14	0.36	No significant terms	-	-	12	-	-
East, 2013	Viral load (DNA copies per ng host DNA) (log10 scale)	LME	4.5	0.033 *	Month (factorial)	5.25	0.008 **	100	23.53%	Processing Batch (11): 22.92%; Sample location (4): 53.54%
	Weight (mg) (log10 scale)	LME	1.56	0.21 ns	Month (factorial)	8.68	<0.001 ***	100	11.23%	Sample location (4): 26.81%
	Wing length (mm)	LM	2.63	0.11 ns	No significant terms	-	-	100	-	-
	Number of insects caught (log10 scale)	LM	2.62	0.14 ns	North/ South	21.78	0.001 **	1, 10	65.39%	-

APPENDIX 7: CHRISTMAS CAROLS

WE THREE KINGS OF INSECT RADAR:

We three kings of insect radar,
Watching a screen in the back of a car,
Field and hedgerow, tracking where bees go,
Hoping they won't go too far.

Chorus:

Radar of wonder, turning free,
Show us where to find a bee,
With transponder, track her yonder,
'Til she hides behind a tree.

Try to track a bumblebee queen,
On a journey that's seldom seen,
Red light flashing, PC crashing,
We stare at a frozen screen.

[Chorus]

Aerial on the back of a drone,
Searching for mates he travels alone,
Dish stops turning, something's burning,
All of the fuses have blown.

[Chorus]

Investigating route formation,
Find a path 'tween feeding stations,
But she's out of range, she's acting strangely,
Maybe she took a vacation.

Joseph Woodgate
December 2016

SILENT NIGHT, MOTH IN FLIGHT:

Silent flight, moth in flight,
Insectary, bathed in red light,
Round yon flight mill, glued to a wire,
Always rotating until he gets tired,
To see how far he'd migrate,
If he was free to migrate.

Silent flight, left and right,
Surrounded by walls of white,
Beating her wings, yet staying in place,
Sensors tell what direction she'll face,
In simulated flight,
Only simulated flight.

Silent flight, kilometres high,
Helped by wind, thousands pass by,
Vertical looking radar can tell,
How high, how fast, what direction as well,
All shown as lines on a screen,
On an oscilloscope screen.

Joseph Woodgate
December 2016

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