Can we extend lifespan *in Drosophila* by changing gene expression or by drug treatments?

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ABSTRACT

Aging is a complex process involving physiological and functional changes in cellular activities, genetic pathways, and overall organismal integrity. The increasing global population and longer life expectancy have indeed created a growing urgency to find ways to extend health span. Considering health span not solely, life span is crucial to reduce the social and economic burden of aging populations. Number of years a person lives in good health free from any chronic disease and disability is more important that the total number of years he lived. A longer life accompanied by more years of chronic illness, frailty, and dependency leads to escalating medical expenses, long-term care needs, and increased public spending. This means not just adding years to life but adding healthy years is important.

Studying gene expression is a powerful tool to understand aging. Ideally, aim of this study was to overexpress a specific gene at defined times within an organism. The GAL4 GeneSwitch system, a modified version of the UAS/GAL4 system, allows for such control over gene expression. It uses the drug RU486 to activate a modified GAL4 protein, enabling both spatial (tissue-specific) and temporal (time-controlled) gene regulation. This system can be used to manipulate genes potentially relevant to aging. In this study, GeneSwitch system was employed to investigate the effects of gene manipulation on lifespan and climbing speed in male fruit flies (*Drosophila melanogaster*). Glyceraldehyde-3-phosphate dehydrogenase (*gapdh 1*) is known for its role in glycolysis and is called moonlight protein because of its multifunctional activities as it acts as a transcription factor and influences cell survival and proliferation. It was used in this research because its expression decreases with age quite a lot, both sexes, and it's expressed ubiquitously and fairly high levels (Flybase). In the first experiment, the Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene was overexpressed under the control of tissue-ubiquitously expressing promoters (*Actin* and *daughterless*). Although *gapdh* was not overexpressed significantly in the experiment. So, overall lifespan was not significantly extended, flies carrying only the UAS- *gapdh* transgene (without the GeneSwitch driver) did show a longer lifespan. Interestingly, flies with the GeneSwitch driver alone (*ActinGS-255B*) displayed increased activity compared to another fly strain (*daughterless*). The results showed no increase in lifespan of flies. These results highlighted the importance of further research into the *gapdh* gene and further explanation of its role in ageing.

Anti-aging drug therapy is considered one of the most promising strategies to tackle the effects of ageing. Combination of different drugs can extend healthy life span when they target age-related mechanisms. In the second project, four different drugs (alagebrium, baicalein,

lithium, bathophenanthroline disulfonic acid), individually and in combination were used to investigate their effect on the lifespan of two wildtype fly strains (Lancaster and Dahomey). 1) Alagebrium is known as cross link breaker formed by advanced glycation end products (AGEs) which are linked to aging. 2) Baicalein is a flavonoid with antioxidant, antibiotic, and free radical scavenging properties. 3) One of the most significant roles of lithium is inhibition of glycogen synthase kinase-3 (GSK-3) which is a tau protein kinase-1 which regulate tau phosphorylation in Alzheimer disease and reported to extend lifespan of *Drosophila* by inhibiting glycogen synthase kinase-3 (GSK-3) via activation of transcription factor nuclear factor erythroid 2 related factor (NRF-2). 4) Bathophenanthroline disulfonic acid (BPS) is an iron chelator that reduces lipid peroxidation, a damaging process in cells. The results showed no significant impact on lifespan for most drug combinations. However, a combination of three drugs (alagebrium, lithium, and baicalein) did lead to a slight increase (3%) the median lifespan of flies from the Dahomey strain ($p=0.05$). In wDah flies, lithium itself significantly increased the median life span of wDah flies by 3% and significantly extended the median lifespan of Lancaster flies by 6% to 67 days ($P<0.05$). The results suggest that these drugs and their combinations did not increase lifespan drastically and they are not worth pursuing, and that the papers using lithium overestimated its effects. As well as it will likely a step towards using novel drugs to extend healthy life span in future.

1. LITERATURE REVIEW

1.1 Aging

Aging is far more than the simple passage of time, it is defined as the progressive decline in an organism's physiological integrity, characterized by a loss of the homeostatic mechanisms that maintain life (Lopez-Otin et al., 2013). This process can also be characterized as a time-dependent deterioration of biological structure, resulting in decreased fertility and an increased risk of mortality (Rodríguez-Rodero, 2011). Aging serves as a major risk factor for chronic conditions, including cancer, cardiovascular disorders, diabetes, and neurodegenerative diseases (Lopez-Otin et al., 2013). However, the underlying, specific causes driving this complex biological decline remain challenging to fully elucidate.

The ultimate goal of ageing research is not merely the addition of years to life, but the addition of life to years. This concept is known as the compression of morbidity, posits that the ideal intervention delays the onset of age-related decline so that it occupies a minimal fraction of the total lifespan (Fries, 2002). Central to the pursuit of morbidity compression is the identification of the Hallmarks of Ageing, which is a set of conserved biochemical markers ranging from genomic instability and telomere attrition to the loss of proteostasis and deregulated nutrient sensing (Lopez-Otin et al., 2013). Hallmarks of aging have been grouped into three categories, 1) Primary hallmark that cause impairment of cellular functions including Telomeric attrition, genomic instability, loss of proteostasis and epigenetic alterations which affect negatively on aging. 2) Antagonistic hallmarks affect based on their intensity including cellular senescence and deregulated nutrient sensing. 3) Integrative hallmarks that negatively impact on tissue function and homeostasis such as stem cell exhaustion and altered intercellular communication (Lopez-Otin, 2023).

It is a long history of fruit flies to be used as a model organism for the identification of genes involved in human diseases. *Drosophila melanogaster* and humans share many ageing-relevant biological and physiological pathways including oxidative stress, insulin-like signaling and DNA repair (Guarente and Kenyon, 2000). *Drosophila* has been considered an excellent model organism to study complexity of aging (Rossi and Gonzalez, 2015). It is a suitable model organism for evaluation of anti-aging compounds (Jafari, 2010).

There are several reasons to use *Drosophila* as a model organism to study aging. They have a relatively shorter life span of three months, altered life span by genetic and environmental manipulation, already published information related to aging, easily available stocks with altered

genes and full genome sequence (Holzenberger et al., 2003). Estimated 70-75% of human disease genes have homologs in *Drosophila*. Moreover, *Drosophila* is versatile and widely used model organism in biomedical research and easy to culture and manipulate in laboratory conditions (Adedeji, 2017) and its genes are 60% orthologs to mammals. As well as it does not require much effort and cost for its maintenance and reproduction (Staats et al., 2018).

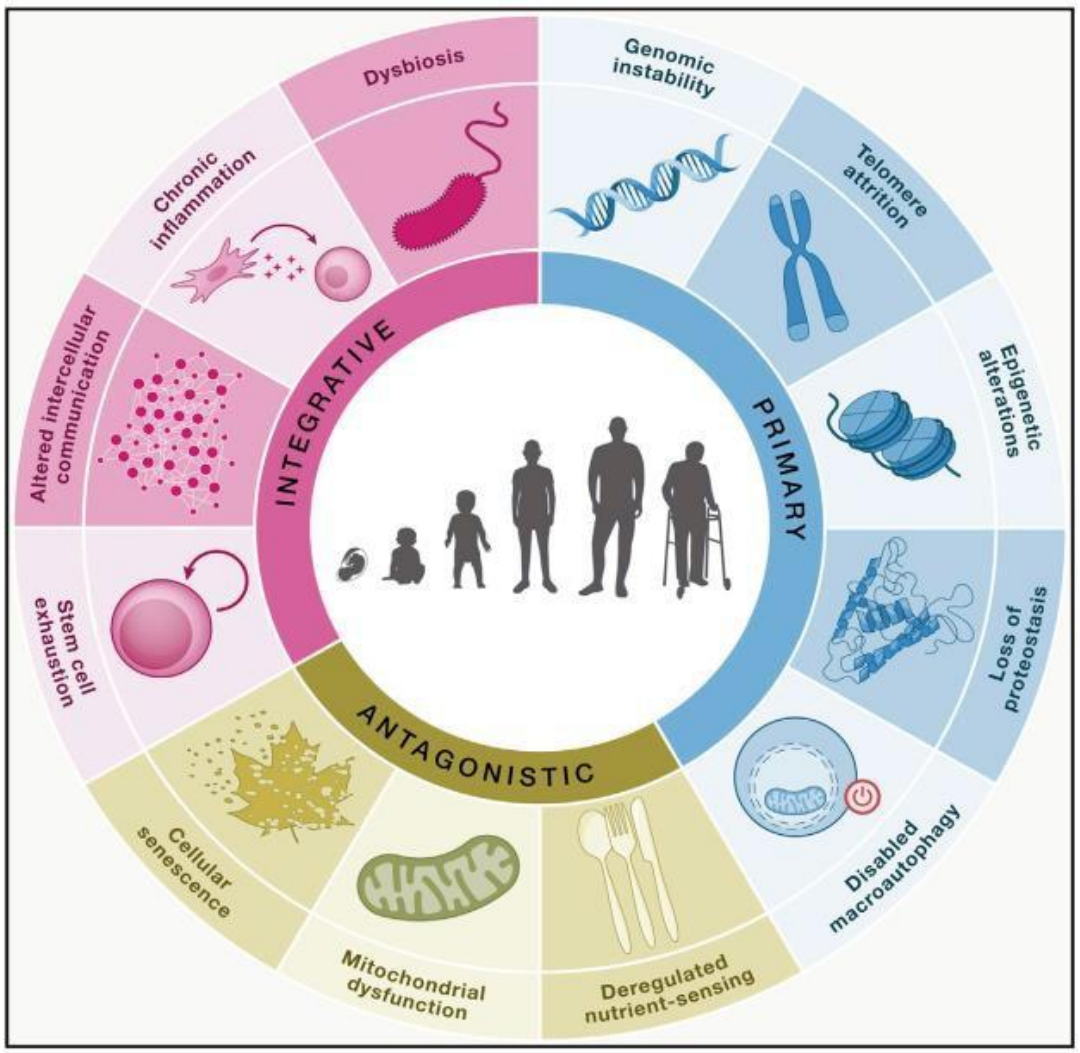

Figure 1 : Hallmarks of Aging: There are 12 hallmarks of aging described through the figure (Lopez-Otin, 2023).

1.1.1 Genetic manipulation and gene overexpression that extended lifespan

Life span of an organism can be modulated by altering gene expressions (Van Raamsdonk, 2018). Some genes associated with ageing regulate different metabolic pathways and extend life span of organisms (Parker and Blake, 1998). Consequently, studying altered gene expression is recognized as one of the most effective techniques for investigating the mechanisms of aging (Iliadi et al., 2012a). This approach is exemplified by the discovery that a single gene mutation can be responsible for dramatically extended lifespan in classic model organisms, including yeast, the fruit fly (*Drosophila melanogaster*), the nematode worm (*Caenorhabditis elegans*), and the mouse (Bitto et al., 2015). Many experimental studies of model organisms have suggested that a single mutation of a gene can increase longevity and delay aging and age-related changes (Iliadi et al., 2012b). And these genetic variations modulate a broad spectrum of physiological processes, including reproductive fecundity, metabolic homeostasis, and oxidative stress resistance, ultimately culminating in significant lifespan extension (Kenyon, 2001).

Substantial evidence suggests that aging in model organisms is hormonally regulated by the evolutionarily conserved insulin/IGF-1 signaling (IIS) pathway (Tatar et al., 2003). The IIS pathway serves as a critical nutrient sensing mechanism that couples environmental conditions such as food availability to growth, metabolism, and lifespan (Skorokhod et al., 1999). The pivotal link between IIS and longevity was first identified in the nematode *C. elegans*. As mutations in this pathway were initially identified for their role in dauer formation, during unfavorable environmental conditions, *C. elegans* can enter into a diapause state, characterized by developmental arrest, reproductive immaturity, and enhanced stress resistance. So, reduction in IIS triggers metabolic shifts toward fat storage and cellular preservation, allowing the organism to postpone reproduction until conditions improve (Kenyon et al., 1993). The IIS pathway operates as part of an integrated nutrient-sensing network that dictates a fundamental metabolic decision: to consume resources for growth and proliferation or to conserve energy by downshifting metabolism (Min et al., 2024). While the IIS pathway communicates systemic nutrient abundance via signals like insulin, it is functionally linked to the target of rapamycin (TOR) kinase network (Kaeberlein et al., 2005). The TOR network and the energy sensor AMPK operate intracellularly, with TOR promoting growth when nutrients are abundant, and AMPK inhibiting growth when energy is low. These two pathways are opposing and interlinked, responding directly to the immediate availability of amino acids and the cellular energy status (González et al., 2020; Jia et al., 2004).

Its pivotal role in longevity was dramatically revealed by mutations in *daf-2* and *age-1* (IIS components) in *C. elegans*, which extended the adult lifespan by 200% through enhanced stress resistance (Guarente and Kenyon, 2000). Mutation that reduces the activity of *daf-2*, a gene encoding an insulin/IGF-1 receptor homologue, significantly extends the lifespan of the *C. elegans*, allowing it to remain active and youthful for more than twice its normal duration (Larsen et al., 1995). Reduced IIS activity enhances the function of the *DAF-16* transcription factor, a member of the FOXO family which regulates genes involved in stress response, antimicrobial defense, and metabolism in *C. elegans* (Murphy et al., 2003). Mutation in *age-1* and insulin-like receptor *daf-2* gene extended mean life span of *Caenorhabditis elegans* by 23 and 35 days respectively (Dorman et al., 1995) and this mutation depends upon *daf-16* which is FOXO family transcription factor (Friedman and Johnson, 1988). The *daf-2* and *age-1* (Insulin receptor) mutants extended life span in flies, worms and mice (Piper et al., 2008).

The link between reduced Insulin/IGF-1 Signaling (IIS) and increased longevity originally identified in *C. elegans* was subsequently also confirmed in *Drosophila melanogaster*. specifically, mutations in the *Drosophila* insulin receptor (*InR*) (Tatar et al., 2001) and mutation in *Chico* gene in *Drosophila Melanogaster* (Clancy et al., 2001). Mutation in *Chico* gene in *Drosophila Melanogaster* that encodes an insulin receptor substrate (IRS) protein which is a key player in the insulin/insulin-like growth factor (IGF) signaling pathway, extended median lifespan of flies in homozygotes and heterozygotes up to 48% and 36% respectively (Clancy et al., 2001). However, the core insulin/IGF-1 signaling (IIS) pathway remains conserved from invertebrates to mammals, the mammalian system exhibits significantly higher complexity (Taniguchi et al., 2006). Mammals utilize three primary ligands, insulin, IGF-1, and IGF-2 which interact with three distinct tyrosine kinase receptors: the insulin receptor (IR), the IGF-1 receptor (IGF-1R), and the orphan IR-related receptor (IRR) (Kornfeld, 1992). Additionally, a unique IGF-2 scavenger receptor serves to modulate ligand availability. And Upon activation, these receptors phosphorylate substrates like IRS and Shc, triggering two diverging cascades: the PI-3K-PKB/AKT pathway, which governs metabolic homeostasis, and the Ras-MAPK pathway, which primarily drives mitogenic processes and cell growth (Taniguchi et al., 2006). The Phosphoinositide 3-kinase (PI3K) pathway is the primary mediator of the metabolic and anti-stress effects of insulin and IGF-1. In the context of aging, this pathway acts as a nutrient sensor that, when overactive, accelerates senescence (Lopez-Otin, 2023).

The activation of the PI3K-Akt/PKB pathway is traditionally viewed as the metabolic arm of the system, yet its true significance in aging research lies in its ability to silence the cell's internal defense systems (Engelman et al., 2006). In the context of aging research, the most

critical downstream target of Akt is the FOXO (Forkhead box O) family of transcription factors (FOXO1, 3, 4, and 6). Under high IIS activity (nutrient abundance), Akt phosphorylates FOXO, leading to its nuclear exclusion and degradation. However, when this signaling is attenuated either through the dietary restriction (Johnson, 2013) or the genetic interventions FOXO is liberated (Brunet et al., 1999) seen in the long lived Ames and Snell dwarf mice which possess mutations in the *Prop1* and *Pit1* genes respectively, exhibit profound deficiencies in Growth Hormone (GH) and IGF-1, these mice consistently demonstrate a 40–50% increase in lifespan and delayed onset of age-related diseases (Bartke et al., 2001). Once in the nucleus, FOXO initiates a robust survival response, upregulating MnSOD for oxidative stress resistance (Kops et al., 2002) and *GADD45* for DNA repair effectively slowing the molecular clock of the cell (Tran et al., 2002). Longevity can also be achieved by targeting mechanisms that maintain genetic stability, as overexpression of DNA repair genes (*spn-B*, *mei-9* and *mnk*) increased lifespan of *Drosophila* male flies by 40% (Shaposhnikov et al., 2015). Overexpression of *D-GADD45* gene in nervous system significantly extended median life span of *Drosophila* by 77% (Plyusnina et al., 2011). As well as Age-related mitochondrial gene is MnSOD (Superoxide dismutase) which is a major defense against ROS, when overexpressed by using an inducible gene expression system extending the lifespan of *Drosophila* by 16% average (Sun et al., 2002). Moderate overexpression of MnSOD has beneficial effects on life span, however elevated levels of MnSOD have toxic effects (Ford et al., 2007). Simultaneously, the Ras-MAPK pathway serves as the mitogenic arm, translating IGF-1 signals into cellular proliferation and growth. While essential for development, chronic over-activation of this branch in later life is a primary driver of cellular senescence (Campisi, 2013). High MAPK signaling can induce oncogene-induced senescence, where the cell permanently stops dividing to prevent cancer but begins secreting inflammatory cytokines (the SASP, Senescence-Associated Secretory Phenotype), which damages neighboring healthy tissue (Coppé et al., 2010).

A comprehensive understanding of IIS in aging requires its integration with the mechanistic Target of Rapamycin (mTOR), Akt indirectly activates mTORC1 by inhibiting the Tuberous Sclerosis Complex (TSC1/2) (Inoki et al., 2002). As a master regulator of protein synthesis, mTORC1 promotes growth at the expense of repair (Johnson, 2013). The suppression of mTORC1 (either through low IGF-1 signaling or Rapamycin treatment) is one of the first definitive evidence to extend lifespan across species, by restoring reducing mitochondrial oxidative stress and extended lifespan of mice both females and males, 14% and 9% respectively (Harrison et al., 2009).

Interpreting these findings to humans, longitudinal studies of exceptionally long-lived populations have identified specific polymorphisms in the FOXO3A gene that are strongly correlated with centenarians across diverse ethnicities (Willcox et al., 2008). Furthermore, individuals with Laron Syndrome (GHR deficiency), who have extremely low levels of circulating IGF-1, appear to be almost entirely protected against cancer and type 2 diabetes, providing clinical validation for the role of reduced IIS in human health (Guevara-Aguirre et al., 2011). Insulin-like-growth-factors (IGF) and their receptors play a significant role in the control of aging (Hoeijmakers, 2009). Genetic mutation in growth hormone (GH), IGF-1 receptor or down stream of mTOR, AKT and FOXO have been linked to longevity in human (Kenyon, 2010). As well as reducing the activity of mTOR and IIS pathway targeting nutrient sensing can extend lifespan and improve health span in aging (Campisi et al., 2019). Heterozygous mutation in *IGF1 R* gene has been found to be associated with reduced IGF-1 signaling and longevity in Centenarians (Van Heemst et al., 2005; Vitale et al., 2019).

Silent Information Regulator 2 (*Sir2*) is an NAD^+-dependent deacetylase and a member of the sirtuin protein acylase family, which encodes an NAD^+ dependent histone deacetylase, is one of the most widely discussed genes in aging research due to its established role in promoting longevity (Hoffmann et al., 2013). While highly conserved from bacteria to mammals, its functional scope has expanded significantly since its initial discovery in yeast. As, *Sir2* is a key regulator of transcriptional silencing at vital genomic sites in yeast (*Saccharomyces cerevisiae*) (Gasser and Cockell, 2001). Although originally identified as a histone deacetylase, mammalian homologs (SIRT1–7) are now known to target a diverse array of non-histone proteins—such as p53, FOXO, PGC-1α, Ku70, and NF-κB positioning them as critical regulators in the pathology of age-related diseases (Haigis and Guarente, 2006). Many identified sirtuin targets significantly influence longevity, establishing these NAD+-dependent deacetylases as evolutionarily conserved regulators of lifespan (Haigis and Guarente, 2006). By maintaining chromatin silencing and suppressing rDNA recombination, *Sir2* acts to reduce genetic instability (Guarente, 2000). The pro-longevity effect is conserved across species: *Sir2* was the first gene identified to extend yeast's replicative lifespan by 30% (Kaeberlein et al., 1999). Overexpression of the ortholog *sir-2.1,* the ortholog of mammalian *SirT-1* has been shown to extend lifespan of *C. elegans*, often through mechanisms involving the forkhead protein DAF-16 (Schmeisser et al., 2013) and its overexpression in the fat body of adult *Drosophila* extended their lifespan by 13% (Hoffmann et al., 2013).

The targeted overexpression of genes within key signaling pathways specifically those managing metabolic regulation and stress defense consistently yields significant longevity

benefits across species (Ma and Gladyshev, 2017). Overexpression of genes involved in metabolic regulation and stress defense often produces significant longevity benefits. For instance, overexpressing Cu/Zn superoxide dismutase (*sod1*) extended mean lifespan of *Drosophila* by 48% by enhancing reactive oxygen metabolism and alleviating oxidative stress (Parker and Blake, 1998; Sun and Tower, 1999). Similarly, the transcriptional coactivator *PGC-1* transcriptional coactivator which is crucial for energy metabolism and mitochondrial biogenesis has a significant role in energy metabolism and mitochondrial biogenesis extended mean life span in *Drosophila* by 49% by increasing mitochondrial activity (Rera et al., 2011). Maintaining the structural integrity of the genome is equally critical for longevity. *Dicer-2* or *su(var) 3-9* gene overexpression increased median life span of flies by 6.6% by maintaining heterochromatin integrity and suppressing TE (transposable elements) activity as TE activity increase with age, supporting the idea that weakened cellular defense mechanisms are letting these molecular parasites run amok in older cells (Wood et al., 2016).

In mammalian models, signaling pathways that ensure accurate cell division are vital for delaying systemic decline (Baker et al., 2013). The *BubR1* gene plays a critical role in ensuring accurate cell division by functioning in both the mitotic checkpoint and kinetochore-microtubule attachments. By functioning in both these aspects, BubR1 helps prevent errors in chromosome numbers, which can lead to genetic abnormalities and diseases. Overexpression of BubR1 in transgenic mice increased lifespan by 15% by delaying age- related pathologies (Baker et al., 2013).

1.1.2 *GAPDH*, could *gapdh* be involved in ageing?

Glyceraldehyde-3-phosphate dehydrogenase-1 (*gapdh* 1) is well known as a catalytic enzyme in glycolysis, which is involved in the sixth step of glycolysis where it catalyzes the conversion of glyceraldehyde 3 phosphate to 1,3 bisphosphoglycerate in the presence of NAD+ and inorganic phosphate (Sirover, 1999). In addition, it is also called moonlight protein because it performs multiple functions which are often unrelated to its well-known role in glycolysis (Jeffery, 1999). It is a multifunctional protein with distinct cytosolic, nuclear and perinuclear localizations including ER, Golgi and polysomes (Sirover, 2012). It is located on human chromosome 12 and encoded by a single gene specifying a single protein of 37KDa (Bruns and Gerald, 1976).

1.1.3 Functions of *gapdh*

Gapdh involved in various cellular processes unrelated to its role in glycolysis such as increased *gapdh* activity is linked to cell growth and tumor development. And *gapdh* can bind to RNA and DNA, suggesting additional regulatory roles (Nicholls et al., 2012a). It also involved in

subcellular localization (Tristan et al., 2011), tubulin regulation (Jung et al., 2014), maintain telomere integrity by binding to telomeric DNA (Sundararaj et al., 2004), tRNA export (Singh, 1993), membrane fusion and transport (Glaser and Gross, 1995), and DNA replication (Sirover, 1999). *Gapdh* also has a significant role in DNA repair (Kosova et al., 2017), iron metabolism, biosynthesis of histones, receptor-mediated cell signaling (Sirover, 2012). Cell death can be inhibited by *gapdh* by increasing ATP levels through glycolysis and stimulating autophagy-media ted clearance of permeabilized mitochondria (Tristan et al., 2011). *Gapdh* also acts as anti-inflammatory protein against lipopolysaccharide induced sepsis related acute lung injury in mice (Takaoka et al., 2014). Binding activity of *gapdh* with Hsp70 can protect normal cells from pathological factors by affecting integrity of enzymes (Lazarev et al., 2016).

1.1.4 Gene expression of *gapdh* with Age

While often employed as a standard reference gene, the subcellular distribution and expression of *gapdh* are inherently age-dependent (Mazzola and Sirover, 2005). Across various organisms and tissues, *gapdh* expression typically decreases with advancing age. This trend is observed in *Drosophila* (figure 2 and figure 3 from flybase; (Gelbart and Emmert, 2013; Vigelso et al., 2015)), the glycolytic muscles of rats (Lowe et al., 2000). *Gapdh* expression was also seen to significantly decrease in aged muscles of mouse (Takaoka et al., 2014). However, *gapdh* expression is sensitive to the cellular environment and represents a complex response to damage. Its expression increases under stressful conditions, including oxidative stress, radiation damage, and cell starvation (Pierce et al., 2008). Furthermore, its role extends beyond basal metabolism; although expression decreases in acute pancreatitis, *gapdh* overexpression has been implicated in the promotion of regeneration and development of the pancreas following injury in rats (Calvo et al., 1997). These findings highlight that the dynamic regulation of *gapdh* expression is not simply passive but is actively involved in the complex balance between aging, damage, and tissue repair.

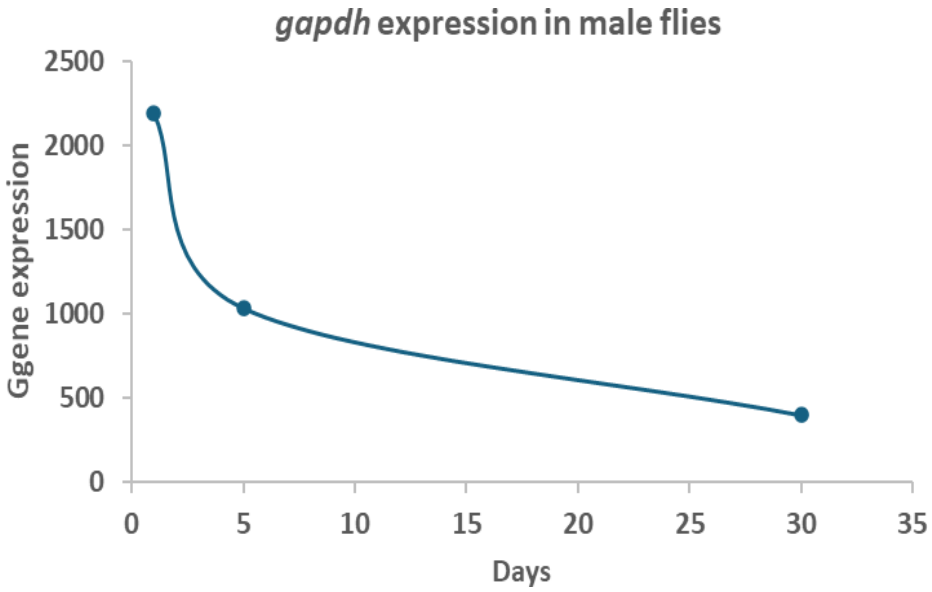

Figure 2: Developmental temporal expression of *gapdh* gene overtime in male flies (flybase was used to find information of gene expression (Gelbart and Emmert, 2013))

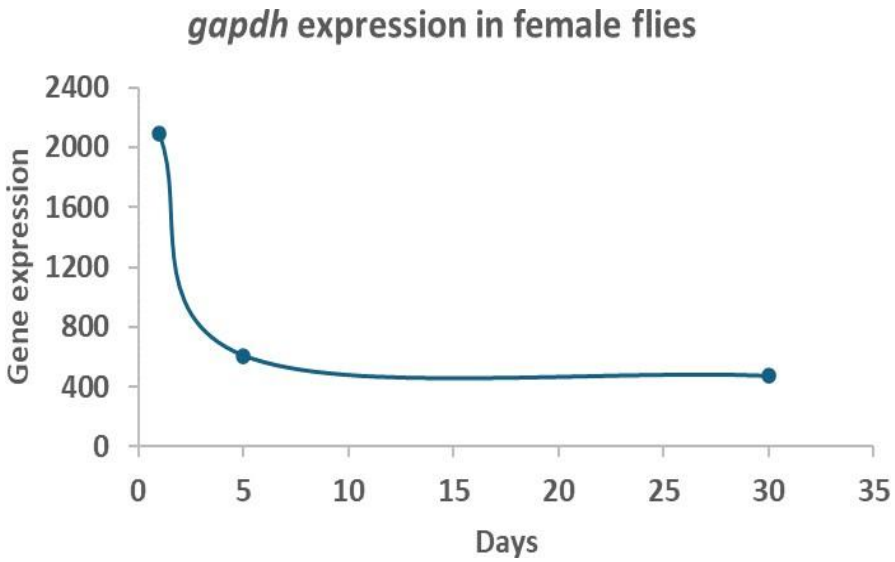

Figure 3: Developmental temporal expression of *gapdh* gene overtime in female flies (Gelbart and Emmert, 2013) flybase was used to find information of gene expression)

1.1.5 GAL4 UAS System

GAL4 UAS system was designed to express a target gene which can selectively activate a cloned gene in a wide range of tissues and cell-specific patterns. This system has two basic components: one is the yeast transcription factor GAL4 which is placed under the control of a ubiquitous or tissue-specific promoter and other is a gene of interest, cloned downstream of a UAS (upstream activating sequence) to which GAL4 binds (Brand and Perrimon, 1993). Flies carrying UAS-transgene crossed with the fly expressing GAL4 genes in specific tissue will then produce transgene-expressing progeny (Miratul and Mel, 2002). This can only be possible if transgene contains GAL4 binding site (UAS- upstream activating sequence) in its promotor that allows this to activate only in the cell expressing GAL4 (Brand and Perrimon, 1993). Mechanism is presented in figure 4.

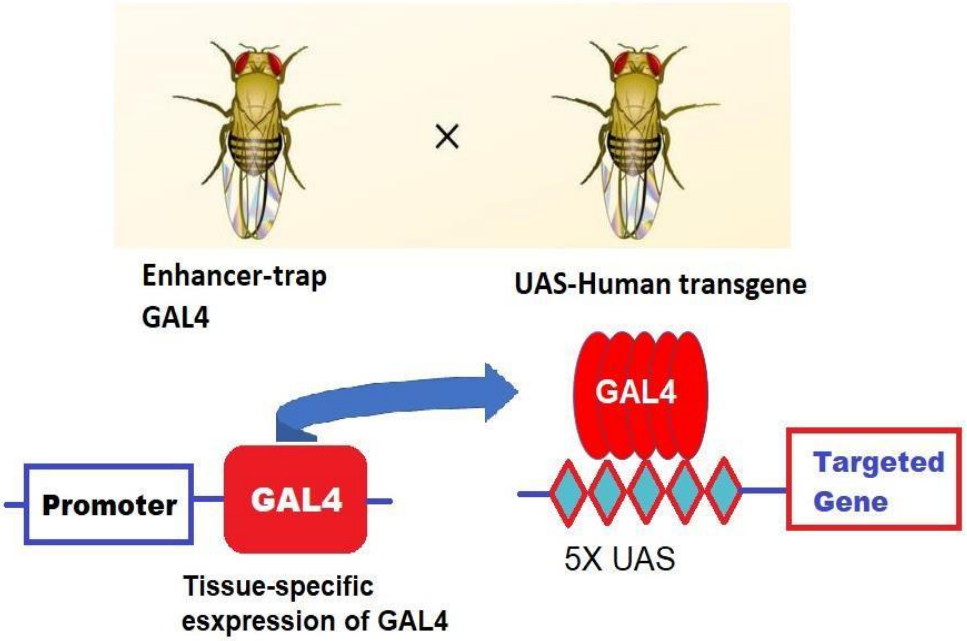

Figure 4. GAL4/UAS System mechanism. Adaptation from Muqit and Feany, 2002

1.1.6 Gene switch system

Gal4 was identified in yeast as a gene regulator which encodes 881 amino acids (Laughon et al., 1984). The GeneSwitch system is a refined, modified version of the UAS-GAL4 system approach that allows for tight temporal and quantitative control over gene expression. GeneSwitch system regulates and induces transgene expression using the synthetic drug RU486 (mifepristone). GeneSwitch requires RU486 to activate transcription, while Gal4 is constitutively active. Unlike the standard Gal4, which is always active, gene switch remains inactive until the synthetic ligand RU486 is administered, allowing for temporal control (Elliott and Brand, 2008). Unlike the standard Gal4, the GeneSwitch protein remains inactive until the ligand RU486 is administered, enabling precise temporal control as well as level of expression of transgene can be controlled by the dose of Steroid (RU486) (Osterwalder et al., 2001). The system's regulatory power is based on two key features: first is expression can be turned on and off by the addition or removal of RU486. And second is the level of transgene expression can be modulated by the dose of the steroid (RU486) (Osterwalder et al., 2001). This inducible control is achieved when a cross is performed between a GeneSwitch driver strain and a UAS transgene strain. RU486 acts as an activator; in its absence, the GeneSwitch-GAL4 protein cannot bind to the UAS element, preventing transgene expression (Osterwalder et al., 2001). The ability to control expression post-development is particularly valuable in aging research, as it removes negative fitness effects that might arise from altered gene expression during pre-adulthood. Although some GeneSwitch lines exhibit low basal activity (expression without RU486), more than 90% of neuron-specific driver lines identified showed no such background activity meaning some activity of the gene switch GAL4 even in the absence of RU486 (Nicholson et al., 2008). Gene switch mechanism is presented in figure 5 which is adaptation from (Osterwalder et al., 2001).

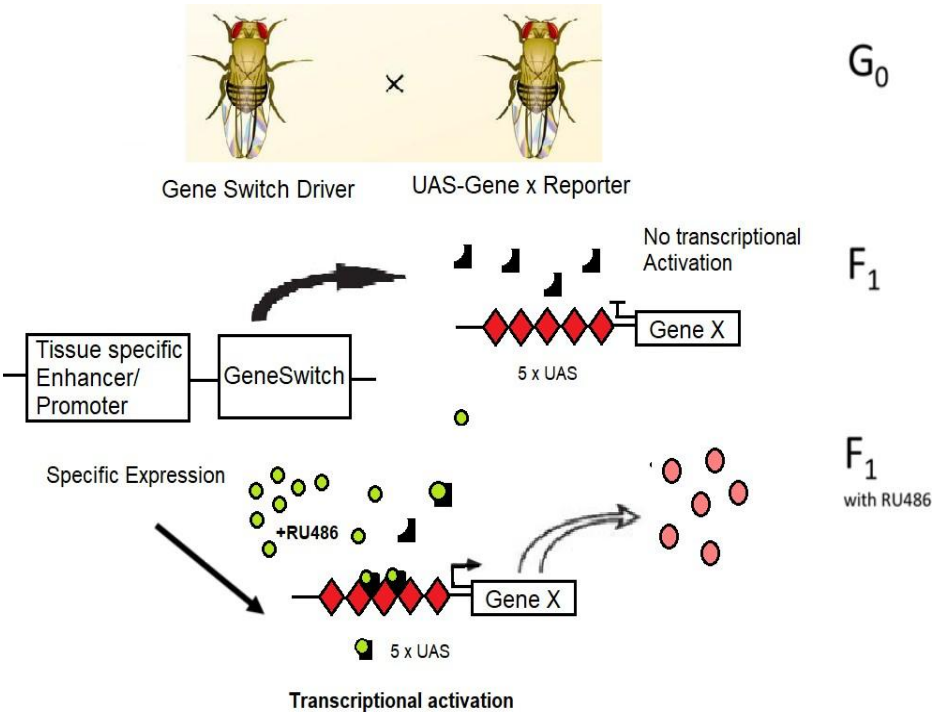

Figure 5. Gene Switch System Adaptation from (Osterwalder et al., 2001).

1.1.7 Limitations of the system

Quantitative analysis of RU486 fed flies showed that expression level of transgene is affected by the RU486 concentration as well as the sex and age of the fly. The expression level can vary by sex and strain of the fly. Sometimes, the expression level can be more in tissues than expected. Also, expression was seen in some strains during adulthood and development of flies even in the absence of the inducer, these driver strains are called leaky (Poirier et al., 2008). It is accredited that this gene switch system is known for its potential for leakiness, manifesting as low-level transgene expression without the inducer RU486. The GS system, while useful, is not perfectly controlled; even without the inducer RU486, which normally activates the modified GAL4 protein, some UAS transgenes are expressed at a low level. Leakiness varies depending upon the transgene type. Leaky expressions seem imperceptible when strong drivers are used. Further controls depending on driver strength and transgene expression are recommended to investigate the extent of leakiness in the strain (Scialo et al., 2016). Gene switch system users must be careful while investigating the promoter effects because of the reasons that some drivers might not be ubiquitous tissue. It is recommended, non-transgenic control with or without drug should include with each driver in the experiment (Andjelkovic et al., 2016).

1.1.8 RU486 effects

In gene switch system, spatial and temporal control of desired gene is facilitated by supplementation of mifepristone (RU486). RU486 itself can affect longevity of both females and males that may be diet and dose dependent. It was suggested that RU486 has bad taste that can decrease food consumption which can reduce longevity (Yamada et al., 2017).

1.2 Drug treatment

There is increasing interest about pharmacological interventions because it targets aging process directly as it is easy to optimize timing and doses of drugs, and it is quite simple to combine drugs (Levine and Cagan, 2016). Triple combination of three different drugs (trametinib, rapamycin and lithium) promoted longevity in flies by 48% by targeting the components of nutrient sensing network such as by reducing the insuline/insuline like signaling (IIS) pathway activity and mechanistic target of rapamycin (mTOR) nutrient sensing signaling network (Castillo-Quan et al., 2019). Some non-steroidal anti- inflammatory (NSAIDs) drugs (Aspirin, valeryl salicylate, APHS, CAY10404, SC-560, NS-398 and licofelone) significantly extended median life span of *Drosophila* male flies by 8.3-19.6% and median lifespan of females by 5.9-12% with the concentration of 0.5 µM. And with the concentration of 1 µM, aspirin increased the median lifespan of female flies by 32.7% and Valeryl salicylate, APHS, CAY10404, SC-560, NS-398 and licofelone increased

median lifespan by 9.6-15.4%, these Drugs increased lifespan by increasing stress resistance, increasing locomotor activity but decreased female fecundity (Danilov et al., 2015). Healthy lifespan can be extended by treatment of dugs combination targeting age related pathways (Admasu et al., 2018). Some of the drugs are mentioned in the table 1 that increased life span of flies by targeting age related Sopathways.

Table 1. Drugs that extended life span by targeting different age-related pathways

Drugs	Effects	Model organism	Reference
Minocycline	Increased lifespan by 33 days by downregulating MDA formation and decreasing motor activity by inhibition of ROS.	Male wildtype *Drosophila*	(Bonilla et al., 2012)
Minocycline	Increased resistance to oxidative stress via FOXO and increased median life span by 26.7%.	Male and female flies of Canton S	(Lee et al., 2017)
Torin1	Inhibition of TOR mechanism and extended life span up to 60% over the control flies.	*Drosophila*	(Mason et al., 2018)
Lithium Chloride	Inhibit glycogen synthase kinase-3 (GSK-3) by activation of the transcription factor nuclear factor erythroid 2-related factor (NRF-2). 1mM of dose extended median lifespan of male flies by 5% and maximum lifespan by 13%. And 25 mM lithium increased median lifespan by 9% and 10mM dose of lithium increased maximum lifespan of female *Drosophila* flies by 4.5%	Male and female *Drosophila*	(Castillo-Quan et al., 2016)
Rapamycin	Increased median lifespan of male flies by 7% and maximum lifespan of female flies by 33% by inhibiting PI3K and TOR-kinase	Male and female *Drosophila*	(Moskalev and Shaposhnikov, 2010)
Rapamycin	Downregulation of mTOR mechanism and extended maximum lifespan by 10%.	*Drosophila*	(Bjedov et al., 2010)

2,5-dimethyl-celecoxib	Extended life span by 8% via mechanism involved in insulin signaling and target of rapamycin signaling.	*Drosophila* (Dahomey) Females	(Wu et al., 2017)
N-acetyl-L-cysteine	Increased median life span by 7.4% and maximum by 10.9% by increasing stress resistance and locomotor activity.	*Drosophila* males	(Shaposhnikov et al., 2018)
Quercetin	Extended mean lifespan by 15% by signaling pathways modulation and resistance to oxidative stress.	*C. elegans*	(Kampkotter et al., 2008)
Fucoxanthin	Increased median lifespan of flies by 11% and mean lifespan of *C.elegans* by 14% by increasing locomotor activity and resistance to oxidative stress.	*C. elegans* and Drosophila	(Lashmanova et al., 2015)
4-phenylbutyrate (PBA)	Altered genes expression and increased histone acetylation and extended lifespan by 60%.	*Drosophila*	(Benzer and Min, 2001)
4-phenylbutyrate (PBA)	Increased locomotor activity and decreased oxidative stress and extended mean lifespan by 33%.	*Drosophila*	(Kang, 2002)

1.2.1 Iron Chelators

Iron is an essential component for living organisms because it participates in most biological pathways including DNA synthesis, cell cycle regulation and oxygen transport. But maintaining a balance is crucial because its excess causes tissue damage and possibly neurodegenerative diseases (Abbaspour et al., 2014). Excess iron can be dangerous, it can cause tissue damage by catalyzing free radical hydrogen peroxide conversion to the extremely reactive hydroxyl radical that can be harmful for protein, DNA and cellular membranes (Emerit et al., 2001). Excess iron plays a significant role in carcinogenesis due to its catalytic ability to increase oxidative stress and induce apoptosis (Toyokuni, 2014; Whitnall and Richardson, 2006). It is associated with the ROS formation by Fenton chemistry which is directly associated with cancer (Toyokuni, 2014). As well as it contributes to tumor initiation and growth (Torti and Torti, 2013). Moreover, it directly deactivates P53 tumor suppressor by downregulating this protein and interferes with P53 and DNA interaction in *Drosophila* (Shen et al., 2014).

Iron retention has been associated with age-related diseases and ageing by participating in lipid peroxidation, blocking genome repair systems and damaging DNA, process called ferroscenescence (Sfera et al., 2018). Iron chelators can reduce tumor cell growth by reducing the intracellular excess iron and have been considered for tumor therapy (Recalcati et al., 2019). Drugs having iron chelating properties worked as neuroprotective in neurodegenerative disease including Parkinson's, Alzheimer's, Huntington's, ALS, and Friedreich ataxia by preventing excess iron contribution to oxidative stress and reduce hydroxyl radicals formation in rats and mice (Youdim et al., 2004).

Iron chelation therapy is used to treat iron overload, a condition where excess iron accumulates in organs like the liver and heart. Each chelator has its own advantages, disadvantages, target diseases, and optimal dosage (Mobarra et al., 2016). Desferrioxamine (DFO) also known as deferoxamine is non-peptide siderophore produced by *Streptomyces pilosus,* mostly studied as iron chelator that has been used to treat and prevent iron overload disease such as β-thalassemia (Chaston and Richardson, 2003). DFO has shown anti-tumor effects in various cancer types both in vivo and in vitro, including neuroblastoma, leukemia, and bladder carcinoma. DFO's anti-tumor activity is likely due to its effects on intracellular iron levels, which are essential for DNA synthesis (Dayani et al., 2004). Aurin tricarboxylic acid (ATA) and deferoxamine (DFO) are both iron chelators. ATA and DFO decreased the viability and proliferation of breast cancer cells (MCF-7). It shows that iron chelation is a promising anticancer strategy due to cancer cells higher iron requirements (Kuban-Jankowska et al., 2017). Deferoxamine can prevent iron mediated oxygen toxicity and chronic and acute inflammatory diseases caused by oxidative stress by excreting iron overload (Emerit et al., 2001). Three iron chelators Deferoxamine (DFO), Deferiprone (DFP) and ICL670 were reported to be clinically important because they can access intracellular iron pools and reduce iron accumulation and ROS formation by labile iron (Glickstein et al., 2005).

Iron accumulation plays a significant role in ageing and senescence. Inhibition of iron accumulation extended the lifespan of *Drosophila* by 21.4% (Massie et al., 1993). Life span of *C. elegans* was extended by inhibiting iron dependent ferroptosis (Jenkins, 2020). Dietary iron supplementation can have deleterious effects on aging by altering oxidative stress rates in adult rats (Arruda et al., 2013).

1.2.2 Bathophenanthroline disulfonic acid (BPS)

Bathophenanthroline disulfonic acid (BPS) is an iron chelator (Missirlis et al., 2006). BPS has also been proved to be beneficial in reducing lipid peroxidation (Knight et al., 1993). Dietary

supplementation of BPS resulted decreased level of systematic iron in *Drosophila* (Yoon et al., 2017). BPS has also been used as iron deficient inducer which results in iron deficient yeast cell formation (Jo, 2009). BPS inhibited DNA synthesis by removing iron from cells which was helpful in the treatment of Chinese hamster lung fibroblasts (CCI 39 Cells) (Alcain et al., 1994). In addition to this BPS alleviated the aluminum toxicity in *Drosophila* flies with chronic dietary overloading of aluminum and extended their median life span dramatically (Wu et al., 2012). BPS increased the life span of Parkin deficient flies up to 5 days, when it was given them in food (Saini et al., 2010).

1.2.3 Baicalein

Baicalein is a flavonoid compound isolated from the root of a chines medicinal traditional herb *Scutellaria baicalensis* which is known for its antioxidant, anti-biotic properties as well as it is a good free radical scavenger (Gao et al., 1999). Furthermore, it also has anti-inflammatory properties (Lin and Shieh, 1996) and it is also known for its anti-viral, anti-allergic properties (Kubo et al., 1984). Baicalein is a strong plant-based iron chelator that can improve iron homeostasis in body and inhibit lipid peroxidation. It can also prevent Fenton chemistry by chelation and radical scavenging mechanism (Perez et al., 2009). It also has anticancer properties. It causes cancer cell apoptosis and cell cycle arrest as well as it also inhibits inflammation and metastasis (Gao et al., 2016b). Dietary administration of baicalein inhibited the growth of human prostate tumors in mice (Miocinovic et al., 2005). It also inhibited ovarian cancer cells without affecting normal cells therefore it can be used in treatment of ovarian cancer (Chen et al., 2013). Furthermore, baicalein can decrease ROS in human carcinoma cells and it also extended the lifespan of *C. elegans* by Nrf2 accumulation and by increasing ARE-dependent luciferase activity and heme-oxygenase-1 expressions in Hct116 cells (Havermann et al., 2013).

1.2.4 Advanced Glycation End products (AGEs)

Advance glycation end Products (AGEs) are heterogenous group of macromolecules which is formed by non-enzymatic reaction of sugar residues and proteins and glycation of lipids, proteins and nucleic acids. Accumulation of AGEs leads to age related heart diseases and acceleration of AGEs has been seen mostly in diabetes mellitus which can result in cardiovascular dysfunction. It is not only limited to diabetic patients, but it also caused heart failure progression in non-diabetic patients as well (Hartog et al., 2007). There are two sources of AGEs for humans, one is formed in body, and the other is consumed with food such as processed food, grilling and roasting (Semba et al., 2010). It was proposed earlier in Maillard theory of aging

that continuous accumulation of AGEs is one of the casual factors of aging (Monnier, 1989). It also affects several hallmarks of aging (Lopez-Otin et al., 2013). Increased AGEs can accelerate age-related diseases (Kim et al., 2017). AGEs also contribute to endothelial dysfunction and arterial stiffening which is considered a significant hallmark of aging (Zieman et al., 2007). Oxidative stress is increased by AGEs when they bind with receptors for advanced glycation end products (RAGE) (Greenwald, 2007). Because the binding AGEs with their receptor can cause many alterations in cell function in normal aging. Receptor of AGE RAGE is also involved in many age-related cardiovascular diseases (Simm et al., 2004).

1.2.5 Alagebrium

Alagebrium is thiazolium derivative and is a first drug that is known as glucose cross link breaker, of crosslinks between proteins (Shapiro et al., 2007). Advance glycation end products (AGEs) increased with aging and form crosslinks of proteins such as smooth muscle cells, endothelial cells and macrophages. Which can lead to the alterations in physiochemical properties of tissues (Bakris et al., 2004). Alagebrium (ALT-711) is useful in protecting heart in aging mice by decreasing AGE accumulation, preventing mtDNA deletion and decreasing oxidative stress (Guo et al., 2009). Increased SOD and glutathione peroxidase activities were seen in alagebrium treated rats. Alagebrium can decrease arterial stiffening and improve endothelial function (Zieman et al., 2007). Furthermore, type-1 diabetic rat heart showed improved myocardial systolic dysfunction when treated with alagebrium. Alagebrium decreased AGE induced intracellular ROS accumulation and increased expression of receptor of AGE (RAGE) (Seo et al., 2015). Alagebrium has been reported to reduce methylglyoxal levels which induce oxidative stress and AGE (Dhar et al., 2016). Also, it also enhances cardiac function in heart patients (Bakris et al., 2004). It can be used to treat age related cardiovascular diseases (Guo et al., 2009). As well as it can improve cardiac function in chronic heart failure patients (Willemsen et al., 2010). It is reported as a promised drug that can slow down ageing or age-related changes (Desai et al., 2010).

1.2.6 Lithium Chloride

Lithium is a drug known to be used to treat bipolar disorder (Chiu and Chuang, 2010) and act as a protector against oxidative and xenobiotic stressors (Lai et al., 2006). Also, lithium gives protective effect against telomerase shortening which is a hallmark of ageing and associated with psychiatric diseases, depression and schizophrenia and oxidative stress (Martinsson, 2013). Neuroprotective ability of lithium includes one of its properties to induce autophagy (Sarkar et al., 2005) which is a cellular process to inhibit cancer (Zhong et al., 2016). Moreover, prostate cancer cell

line proliferation can be inhibited by lithium chloride (Ge and Jakobsson, 2018). Lithium has also been used to rescue structure and morphology of brain mushroom bodies in adult *Drosophila* flies from Cornelia de lange syndrome (Grazioli et al., 2021). One of the most significant roles of lithium is inhibition of glycogen synthase kinase-3 (GSK-3) which is a tau protein kinase-1 which regulate tau phosphorylation in Alzheimer disease (Hong, 1997). As well as low dose of lithium can decrease mortality and can influence anti-aging capabilities in evolutionary distinct species including *C. elegans* and mammals by activation of the transcription factor nuclear factor erythroid 2- related factor (NRF-2) (Zarse et al., 2011).

Beyond its clinical uses, lithium has shown significant potential as an anti-aging agent across various species. Research indicates that lithium can extend the median lifespan of *C. elegans* by 46% (McColl et al., 2008). And significantly increased life span of *Drosophila* female flies when they fed high sugar diet with 1mM lithium dose by blocking high sugar induced triglyceride accumulation (Jans, 2021). Mechanistically, Oral treatment of lithium chloride extended median lifespan by 7% of *Drosophila* associated with inhibiting glycogen synthase kinase-3 (GSK-3) and activation of transcription factor nuclear factor erythroid 2 related factor (NRF-2) (Castillo-Quan et al., 2016). The inhibition of GSK-3 is responsible for decreasing ROS production and DNA damage (Guidotti et al., 2015). Lithium Chloride extended lifespan of *C. elegans,* fission yeast and *Drosophila*. However, this drug has a narrow therapeutic range but can have severely negative effect on kidney health in case of overdosing (Nespital et al., 2021).

1.3 In this Project

This research comprises two distinct projects aimed at identifying genetic and pharmacological interventions that can extend the lifespan and healthspan of *Drosophila melanogaster*. First project investigates the effect of adult-specific overexpression of the Glyceraldehyde-3-phosphate dehydrogenase-1 (*gapdh1)* on fly longevity. The GeneSwitch transgenic system, driven by two ubiquitous drivers (*ActinGS-255B and daughterless*) and their crosses, UAS- *gapdh* x *Actin-GS*-GAL4 and UAS- *gapdh* x *DaGS*-GAL4 will be used to overexpress of *gapdh1* to the adult male flies, thus eliminating developmental confounding effects gene (FlyORF (Bischof et al., 2013)). Two doses of RU486 (1.6µg/mL and 3.2 µg/mL) will be used to examine dose-dependent effects on lifespan, affect the lifespan of flies. RT-PCR will be used to confirm and quantify the level of overexpression achieved.

In the second project, effects of four drugs (alagebrium, baicalein, lithium, bathophenanthroline disulfonic acid) alone and in combinations will be tested on the life span of two wildtype strains (Lancaster and Dahomey) of *D. melanogaster* to find out if they will increase

life and health span. Nacl will be used as control for lithium chloride to ensure that possible lifespan extension is specific to lithium not a general salt (Cl) effect as well as Nacl have no negative effects. Moreover, negative geotaxis will also be performed for both experiments to analyze climbing height of the flies to analyze if health of flies also have been improved or not.

2. Material and Methods

2.1 Overexpressing *gapdh* by Gene switch

2.1.1 Fly preparation

For this project three types of flies of genetic background Dahomey along with two GeneSwitch driver strains (*daughterless* and *Actin 255B*) were used. Dahomey is wild type strain collected in 1970 as a mass-bred strain from their natural habitat called Dahomey, now Benin, west Africa and has been maintained at medium to large population sizes in the lab since that time. *Daughterless* flies were collected in early days of research during 1930s to 1950s. The driver and responder parental strains were originally homozygous but for the experiment were crossed to white Dahomey. Therefore, they were made heterozygous. *Actin-255B* was a kind gift from Prof John Tower. *Daughterless* was a gift from the Linda Partridge lab.

The Experiment was started by setting up the GeneSwitch crosses necessary to induce *gapdh* overexpression. Dark, red-eyed flies were initially collected from the three required parental stocks: *gapdh*, *daughterless* and *Actin-255B.* Then Crosses were done between the females of *gapdh* and males of *Actin-255B* and *daughterless* flies. UAS-*gapdh* (Females) x *ActinGS-255B* (Males), yielding the UAS-*gapdh* x *Actin* line. And UAS-*gapdh* (females) *daughterless*-GAL4 (Males), yielding the UAS-*gapdh* x *Da* line. Parental flies were kept at 25°C in the bottles with mixed sex for allowing them to mate and fed them with active yeast. To ensure experimental uniformity and mitigate the confounding effects of larval crowding on adult health, the egg squirt protocol (Clancy and Kennington, 2001) in which eggs were collected from cages over a short period. Each strain was kept in bottles with 60mL food. After two days cages were settled to collect eggs. For each strain, 2 cages were set up to collect enough eggs. Every cage contained a petri dish which was filled with food of the same recipe as mentioned in section 2.1.2, but it contained more agar (24g of agar per liter of water). A paste of live yeast with water was added in the middle of each petri dish. Then flies were placed inside the cages, they were incubated overnight at 25°C by covering them with a dark paper to give them humid environment and minimum lighting. Then males were collected from both crosses with the procedure explained above and named as UAS-*gapdh* x Actin and UAS-*gapdh* x Da.

On the next day, adult flies were discarded after laying eggs. Then yeast was removed with spatula. PBS (phosphate buffered saline) was used to wash eggs and eggs were gently removed from petri dishes with the help of soft brush. Then the suspension of eggs was poured into a 15mL tube then eggs allowed to settle. To maintain the standardize larval density, equal

aliquots of egg-PBS mixture was pipetted onto plates. This is important to control the larval density in all treatments because it may affect longevity. This wash was repeated twice. Then PBS was discarded and 32 µL of egg suspension (~300 eggs) were placed into bottles containing food (sugar/yeast/agar). Then they were placed in 25°C for 9 days to develop and then emerging flies were collected. Males were collected from driver strains (*Actin 255B* and *daughterless*), and virgin females were collected from UAS-*gapdh*. Two genetic crosses (*gapdh* and *Actin* 255B, *gapdh* and *daughterless*) were done. After seven days, Eggs were collected again by setting up cages. Then only males were collected to set up experiment after three days from their hatching. Only males were used in the experiment is because female *Drosophila* experience significant physiological changes related to reproduction, including fluctuations in body weight due to egg laying, which can confound lifespan measurements. And experiment was so large that we could have made double number of female flies. So only males were used.

A total of 2240 flies were used. For each condition, 8 vials were prepared containing 20 males each. A total of 14 treatments mentioned in table 2 were performed.

2.1.2 Food preparation

For 1 liter of food, bring one liter of water adding 80g yeast, 80g sugar and 16g of agar to boil on an induction hob and then allow it to cool at 65°C then 5mL of propionic acid and 15 mL of nipagin at 10%w/v was added in ethanol. Then let it cool below 60°C and then transfer food to the vials by liquid pump. Then let it dry for 24 hours and stored at 17°C. Food preparation was done every other week to make sure that food is fresh.

2.1.3 RU486 addition

RU486 stock solutions were made 1.6 µg/mL and 3.2 µg/mL of RU486, these two doses were used from previous research (Tower et al., 2017) per vial. Stock solution was stored at 4°C and RU486 was stored at 11°C. 17µL of RU486 stock solution was added to the vials 2 days prior to the flies being tipped to the vials to ensure that it was dried before the fly's exposure to it.

2.1.4 Lifespan Assay

70 µL of ethanol was used as control. RU486 was given to the flies after 14 days of experiment set up. Food used was prepared with the same recipe. Vials were changed 3 times a week. Dead censored (when flies accidentally escaped or crushed) and transferred dead flies were recorded in every tipping. Data for this life span section was analyzed by using IBM SPSS (IBM Corp., IBM SPSS statistics for Macintosh, version 24, Armonk, NY).

Table 2: Treatments used in *gapdh* experiment.

Control (No RU486)	**1.6 µg /mL of RU486**	**3.2 µg /mL of RU486**
UAS- gapdh x DaGS	*UAS- gapdh x DaGS*	*UAS- gapdh x DaGS*
UAS- gapdh x ActinGS	*UAS- gapdh x ActinGS*	*UAS- gapdh x ActinGS*
Daughterless (DaGS)	*Daughterless (DaGS)*	*Daughterless (DaGS)*
ActinGS	*ActinGS*	*ActinGS*
UAS- *gapdh* x *DaGS* (Antibiotic)		
UAS- *gapdh* x *ActinGS* (Antibiotic)		

2.1.4. Antibiotic treatment

Antibiotic (50 µg/mL chloramphenicol) was added only two times (first when flies were 30 days older and second when they were 48 days older) in the flies life span by mixing it in their food. Antibiotic was given to the crosses of flies (UAS- *gapdh* x *Da* and UAS- *gapdh* x *ActinGS*). Treatments were set up with antibiotic (50 µg/mL chloramphenicol) to safeguard flies from bacteria and to see if there is any effect on their life and health span.

2.1.5 Negative Geotaxis

Negative geotaxis was done four times during first experiment, before giving them drug when they were 12 days old, and after that 3 times on the day 26, 34 and 49. Same vials were measured for negative geotaxis each day. To measure locomotor ability, flies were monitored for their climbing height over a 15 second interval. All flies from each vial were transferred into glass tubes. Tubes were placed in a controlled environment at 25°C and allowed to stabilize for 5 minutes. Flies were tapped to the bottom of the tube (banged down). After 15 seconds of climbing, a photograph was taken to record their positions. This process was repeated three times per treatment group, with a one-minute recovery period between replicates. The resulting images were analyzed using ImageJ software to map the X and Y coordinates of each fly relative

to the tube's base. These coordinates were then used to calculate the specific distance climbed by each fly within the 15 second window.

The mean climbing height was figured out for the flies in each individual photograph. These values were then, averaged across the three photos for each tube, providing a robust mean climbing height per tube. To assess if there were significant differences in climbing performance between different treatments, an Analysis of Variance (ANOVA) performed using SPSS software. The calculated means were used to generate graphs illustrating the climbing performance of flies from different treatments.

Figure 6. Negative Geotaxis experiment set up

2.1.6 RT-PCR

Four flies from each treatment were used to perform expression analysis of *gapdh* gene. First, RNA extraction was performed. Then cDNA was synthesized. Then RT-PCR was performed.

Step 1. RNA Extraction:

For RNA extraction, four flies were selected form each treatment and these flies were placed in Eppendorf tubes and submerged into liquid nitrogen. These were then stored in -80^{0}C. RNA extraction was performed by using Norgen Biotek total RNA purification Kit with following steps:

Flies were taken out from the freezer in a cold box. Bashing beads tubes (provided in the kit) were placed in cold box and 600 µl of lysis buffer was added then flies were carefully tipped into tubes containing lysis buffer and then placed into RiboLyser for two sets of 30 seconds. Then again placed on ice box, samples were placed onto ice box in between each of the following steps. The samples were spun at 12000 × g for 2 minutes. Then lysate was transferred into RNase-free microcentrifuge tubes. Equal amount of 70% ethanol was added to each sample and vortexed. A fresh spin column (provided) was assembled, and lysate was pipetted onto column and span at 12000 × g for one minute and insured that all lysate was passed through. Flow-through was discarded and the same step was repeated once more.

Then the column was washed by adding 400 µl wash buffer (provided in the kit) to each column and was spun for 12000 × g for one minute and flow-through was discarded and this step was repeated further two time. The last spin was done for two minutes with no added wash buffer. Then, for RNA Elution, columns were transferred to the fresh tubes. 20 µl of Elution buffer (Provided) was added to each column and span for 2 minutes at 2000 × g followed by 1 minute at 14000 × g. Then RNA samples were analyzed for purity and concentration by using Nanodrop 2000c spectrometer (Thermofisher Scientific, USA). Then samples were kept on ice to perform Reverse transcription.

Step 2. 1st strand cDNA synthesis:

After RNA extraction, cDNA was made with the following procedure by using Takara, Bionic, layer France. The following mixture was prepared in microcentrifuge tubes for each sample. 1µl Oligo dT primer was added then 1µl of dNTP (100mM) was added in them and afterwards 8µl of Template RNA total RNA ≤5 µg was added. Then heated them at 65^{0}C for 5 minutes and then cool them on ice. Then reaction mixture was prepared by following: 10µl of Template RNA/Primer mixture, 4µl of 5× prime script buffer, 0.5µl of 100 units of prime script

reverse transcriptase were added to make total volume of 20 µl. Then mix them gently. Then heat them at 42^0C for 30 minutes. Then cool them on ice.

Step 3. RT-PCR performed:

RT-PCR was conducted for DNA treated samples by using *EF1gamma* as a reference gene and *gapdh* as a target gene. Only one reference gene, *EF1 gamma*, was used in the experiment because this gene is very stable and highly expressed across a range of conditions. *Ef1 gamma* has frequently shown low variability in its expression across a wide range of biological samples and experimental treatments in many organisms, including *Drosophila*. This empirical stability makes it a reliable internal control for normalizing RT-PCR data. Because of the lost sample RT-PCR was performed only for *Actin* flies. Master mix was prepared for each gene by adding 5µl of SsoAdvanced SYBR green supermix (Bio-Rad Laboratories, Hercules, CA, USA), 1µl of DNA template, 2µl of dH_2O, 1µl of forward and reverse primers (100mM of each).

Four biological replicates for each treatment were used. Samples were added in triplicate into a 96 well plate by using 9µl of each master mix and 1µl DNA. And one row was used as control by adding 1 µl of dH_2O. Then the plate was centrifuged and run on CFX96 thermal cycler (Bio-Rad C1000) at 95^0C for 30 seconds, then 39 cycles for 95^0C for 15 seconds and then 57^0Cfor 15 seconds.Following primers wereused: *gapdh* Forward: TAAATTCGACTCGACTCACGGT and *gapdh* Reverse: CTCCACCACATACTCGGCTC. EF1g forward: 5'-GTC CCT TCT CTT CTT CGT TTC-3'; EF1g reverse: 5'-AAG TCT ACA GAG TTC CTT TCA CC-3'. Then it was analyzed REST2009 (uses Pfaffl method, sampling with replacement (Pfaffl et al., 2002)).

2.1.7 Statistical analysis

Data analysis for the survival section of the experiment was done using IBM SPSS (IBM Corp., IBM SPPS Statistics for Macintosh, Version 24.0, Armonk, NY). SPSS is necessary for the survival section because it handles time-to-event data and censored data (subjects whose final event time is unknown). It provides specialized tools like Kaplan-Meier survival curves to accurately model and compare survival rates across groups (Kaplan and Meier, 1958). And Tarone-ware test was used to do the survival comparison. Reason why tarone ware test was done is because tarone-ware is a test for comparing the equality of survival distributions. Time points are weighted by the square root of the number of cases at risk at each time point.

In negative geotaxis, ImageJ was used to analyze the pictures taken. ImageJ was used to convert the raw visual data (pictures of climbing performance) into reliable numerical data that could be analyzed by SPSS (Schneider et al., 2012). It allows measuring pixel distance, tracing

subject movement, or tracking heights achieved, which is essential to generate the continuous variable (e.g., distance climbed) required as input for the subsequent ANOVA test (Fisher, 1925).

RTPCR data was analysed with the delta Ct method using REST2009 software. REST2009 (Relative Expression Software Tool) is highly specialized for analysing quantitative PCR (qPCR) data using the delta CT method, but with superior statistical rigor compared to a basic spreadsheet program. provides a precise method for calculating the relative quantification (Fold Change). This avoids the common pitfalls of performing basic statistics on the exponential fold change values in a simple spreadsheet (Pfaffl et al., 2002). For the drug Experiment same software was used to perform survival curves and negative geotaxis as well.

2.1.8 Life span

For the second Project, two strains of flies of genetic background, Lancaster red eyed and Dahomey (WDah) white eyed were used. 8 Cages were settled as same procedure explained in 2.1.1 section to collect eggs. Then males were collected to settle experiment after three days of hatching.

For both strains, a total of 24 treatments were performed with 6 vials per treatment, with each vial containing 20 ales, total 2,880 flies. Treatments were the same as mentioned in table 3. Different combinations of four drugs were used for both strains (Lancaster and Dahomey). Drugs were given to the flies after 15 days of experiment was set up. Food was prepared with the recipe; For 1L of Food: 80g of sugar, 16g of agar, 30ml of Nipagin, 5ml of propionic acid and 80g of yeast. Some drugs were mixed into the food (sugar/yeast/agar), and some were squirted onto the food. Total four drugs named; alagebrium (1.4mg/mL), lithium Chloride (15mM), baicalein (0.2mg/mL), Bathophenanthroline disulfonic acid (80mg/L) and NaCl (15mM) and their combinations were used in experiment. Doses of the drugs were used from existing literature, 15mM lithium chloride from (Castillo-Quan et al., 2016), baicalein (0.2mg/mL) from (Gao et al., 2016a), Bathophenanthroline disulfonic acid (80mg/L) from (Missirlis et al., 2006) and with alagebrium where it was mice we estimated based on estimates of how much food flies eat per day based on (Vasan et al., 2003). Alagebrium, also known as ALT-711 was from (Tokyo Chemical Industry Co) and BPS was from Fisher Chemical.

All drugs were given to the flies by mixing them in their food except alagebrium. 70 µL of alagebrium (1.4mg/mL) was used to squirt onto the food a day before tipping. 70 µL ethanol was also used to squirt on controlled treatment a day before tipping flies. NaCl (15mM) was used as control for lithium chloride. Vials were changed 4 times a week. In every tipping, transferred, dead and censored flies were recorded on spread sheet in Microsoft excel. Data

analysis for this life span section was analyzed by using IBM SPSS software (IBM Corp., IBM SPSS statistics for Macintosh, version 24, Armonk, NY).

Table 3. Drug treatments Used in Experiment.

No	Treatments
1	Alagebrium and baicalein
2	Lithium and baicalein
3	Alagebrium, lithium. baicalein
4	Alagebrium and lithium
5	Alagebrium and BPS
6	NaCl with 70% ethanol
7	Lithium and BPS
8	Alagebrium in 70% ethanol
9	BPS in 70% ethanol
10	Baicalein in 70% ethanol
11	Lithium chloride in 70% ethanol
12	Lancaster control
13	WDah control

2.1.9 Negative Geotaxis in drug experiment

Negative geotaxis were done only once during the life span of drug-treated flies. Flies were 28 days old. This procedure of negative geotaxis was the same as used in the above experiment.

3. RESULTS

This work had two separate projects. The first asked: Does overexpression of *gapdh* during adulthood extend lifespan and health? To investigate this, Gene Switch transgenic system with two ubiquitously expressing driver strains (*Actin* and *daughterless*) and two doses of the RU486 started 14 days after setting up the experiment, testing survival and the decline over time in speed of climbing were used. Drivers without responder and responder without drivers, as controls were used in the experiment. The second experiment assessed the effects of four drugs, alone and in combination, on lifespan of two wildtype strains: Lancaster and Dahomey.

3.1 Overexpression of *gapdh*

3.1.1 Survival

The aim of the experiment was to investigate how the overexpression of *gapdh* during adulthood affected the life or health span of *Drosophila* as well as how RU486 alone affected the lifespan of flies. UAS- *gapdh* flies were 4 days older than Actin and *daughterless* driver strain flies because they emerged late. So, RU486 was applied 4 days later than UAS- *gapdh* x Actin and UAS- *gapdh* x Da. Using a sample of 2,240 male flies, the effects of two RU486 dosages (Low dose = 1.6µg/mL, Hi dose = 3.2 µg/mL) were compared across different fly strains.

Initial results showed that RU486 generally failed to extend lifespan and, in several cohorts, had a detrimental effect. For the *Actin-GeneSwitch* driver, a high dose of RU486 significantly reduced median lifespan by 18% (Tarone ware test, p=0.00), suggesting drug-induced toxicity rather than life-extension. While *UAS-gapdh x Actin* flies showed a slightly higher median lifespan than their drivers (57 vs. 53 days see Figure 7, Table 4 for mean life spans with 90% mortality), the high dose of RU486 still negatively impacted survival. Median life span of UAS- *gapdh* x *Actin* flies and *Actin* driver flies was 57 and 53 days respectively (see Figure 7, Table 4 for mean life spans with 90% mortality).

Actin driver flies showed no lifespan improvement across treatments. Median lifespans for UAS-*gapdh* x *Da* and *Da* driver flies were 49 and 51 days (Table 4). While 3.2 µg/mL of RU486 extended maximum lifespan a little (p=0.03), the low dose had no significant effect according to Tarone-ware test (p=0.08). Overall, RU486 did not provide a clear survival advantage, though it was less toxic to the *Da* driver lines than the *Actin* lines. Overall, the *Actin* driver flies maintained better health and longer lifespans than the *daughterless* group (Figures 7 & 8).

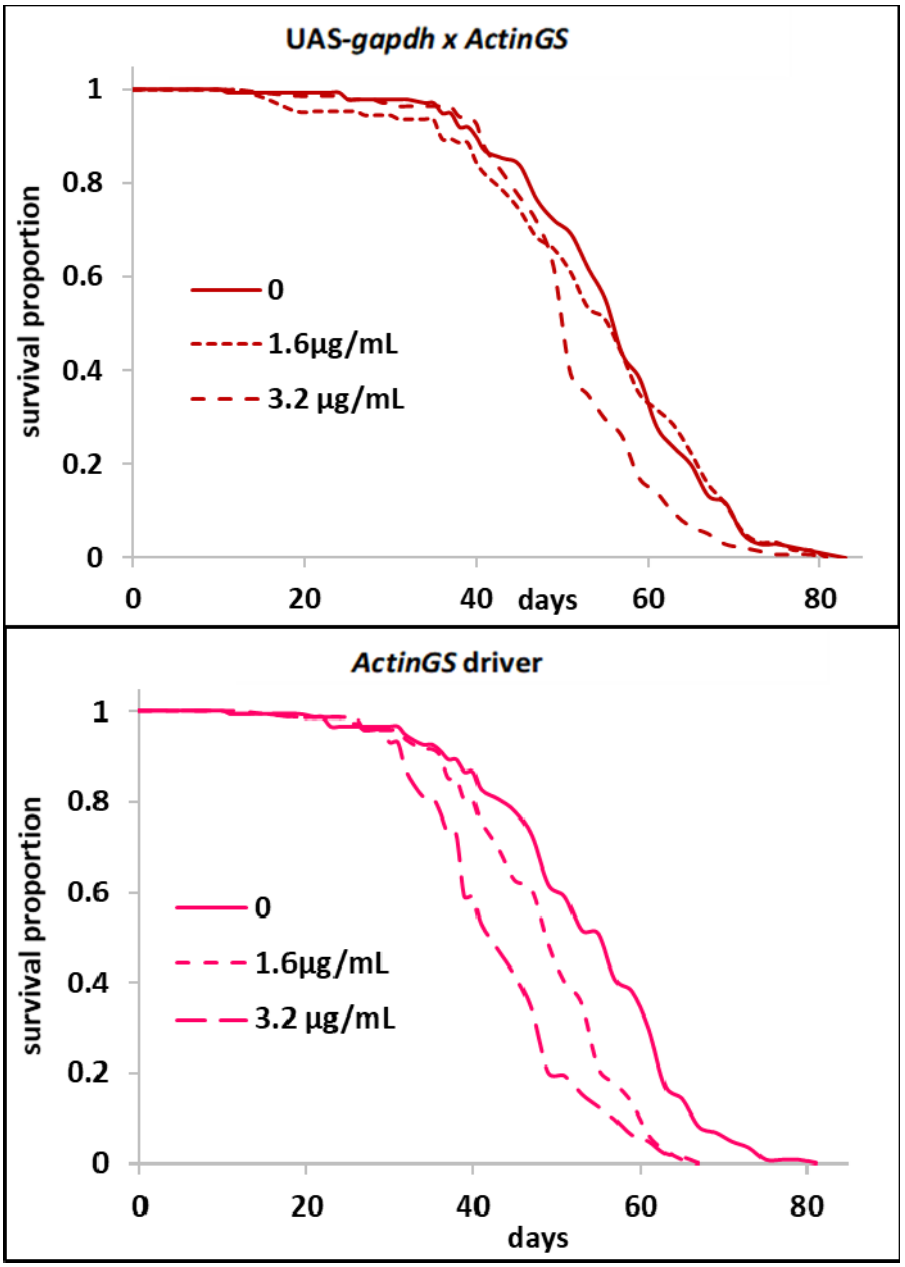

Figure 7. Survival curves for UAS-*gapdh x ActinGS* and *ActinGS* driver control flies across RU486 varying concentrations. Flies were given **3.2** µg/mL, 1.6µg/mL of RU486 to assess the impact of *gapdh* overexpression on longevity. Top panel showing survival proportion of *UAS-GAPDH x ActinGS* flies with median lifespan and sample sizes were; control=57days, N= 96; 1.6µg/mL= 57days, N=86; 3.2 µg/mL= 51days, N= 84. Bottom panel showing Survival proportion of *ActinGS* driver flies with median lifespan and sample sizes were; control= 53days, N= 98, 1.6µg/mL= 49days, N=87; 3.2 µg/mL= 43days, N= 85. Statistical differences between curves were evaluated using the Tarone-Ware test. For the *ActinGS* driver, a clear dose-dependent reduction in survival was observed with both RU486 doses compared to the untreated control. In contrast, the UAS-*GAPDH x ActinGS* line showed increased resilience to the high-dose treatment compared to its respective driver control.

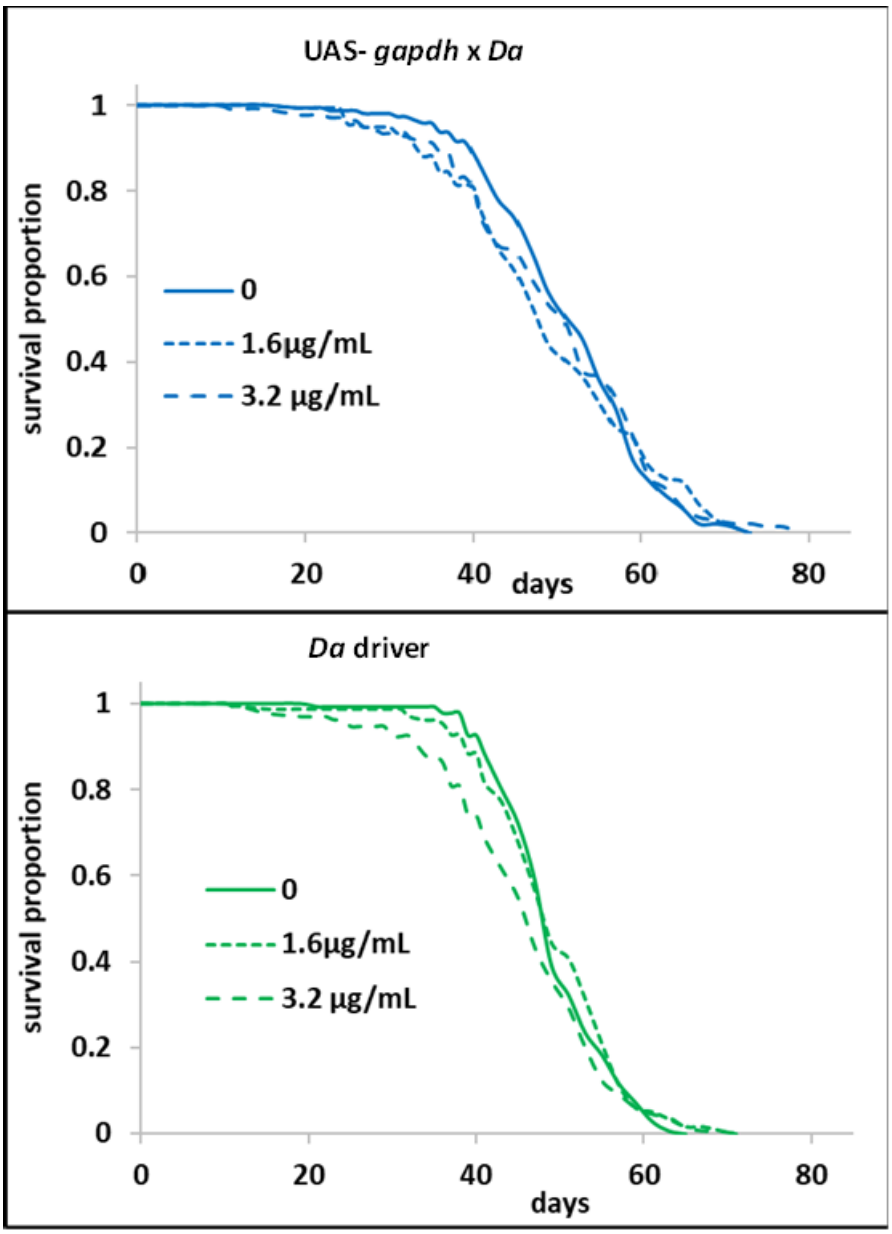

Figure 8. Survival curves for UAS- *gapdh* x *Da* and *Da* driver flies across RU486 varying concentrations. Flies were given 3.2 µg/mL, 1.6µg/mL of RU486 to assess the impact of *gapdh* overexpression on longevity. Survival proportion of *UAS-gapdh x ActinGS* experimental flies. Top panel showing survival proportion of UAS- *gapdh* x Da with median lifespan and sample sizes were; control= 51days, N= 85; 1.6µg/mL= 49days, N= 95; 3.2 µg/mL= 49days, N= 100; Bottom panel showing Survival proportion of Da driver flies with median lifespan and sample sizes were; control= 49days, N= 76; 1.6µg/mL= 49days, N= 68; 3.2 µg/mL= 47days, N= 88. Statistical differences between curves were evaluated using the Tarone-Ware test.

3.1.2 Effects of *gapdh* Overexpression on climbing performance of D.*melanogaster* with age.

Negative geotaxis was done to analyze the climbing speed (height climbed in 15s) of flies. It gives insights into neuromuscular function changes related to aging. Flies from the same vials used in survival experiment (described above) were removed from vial and tested for negative geotaxis. It was done to investigate whether RU486 or overexpression of *gapdh* affected the climbing performance of flies in their life span. As described above, UAS- *gapdh* x Driver flies were 4 days older than *Actin* and *daughterless* driver strains because they emerged later. So, Drug RU486 was applied 4 days later than UAS- *gapdh* x *Actin* and UAS- *gapdh* x *Da* and flies were 4 days of age apart when climbing speed was measured. Negative geotaxis was performed on day 12, 24, 34 and 49 of the survival experiment. Then Repeated measures ANOVA of Climbing height over time was calculated to compare the results of all conditions where triplicate was nested within vials and vials were nested within time and dose. This analysis used vial means as the experimental units.

As expected, climbing height declined over time across all treatment groups. In UAS-*gapdh* x *ActinGS* flies, RU486 treatment showed a dose-dependent benefit: the low dose increased climbing height at day 34, while the high dose improved performance at day 47 (Figure 9).This was supported by a significant interaction (time X cross, $p=5.0x10^{-6}$ and time X cross X dose, $p=1.68 \times 10^{-8}$) according to ANOVA results. Conversely, RU486 decreased climbing height in *Actin* driver flies. While a slight increase was noted at day 22 with the low dose, the overall effect of the drug was negative (Figure 10).

For the UAS-*gapdh* x *Da* line, the high dose of RU486 significantly improved climbing height at day 26, with moderate increases maintained at days 34 and 47. According to ANOVA results there are significant interactions (time X cross, $p=7.6x10^{-41}$ and time X cross X dose, $p=1.75x10^{-7}$) In Da driver flies, RU486 improved initial performance at day 22 and day 30 (Figure 12).

In comparative terms, Da driver flies outperformed *Actin* driver flies regardless of RU486 treatment. However, the UAS-*gapdh* x *Actin* flies were notably more active than the UAS-*gapdh* x *Da* flies. RU486 exerted a positive effect on the climbing ability of Da-associated lines but had a deleterious effect on *Actin* driver flies.

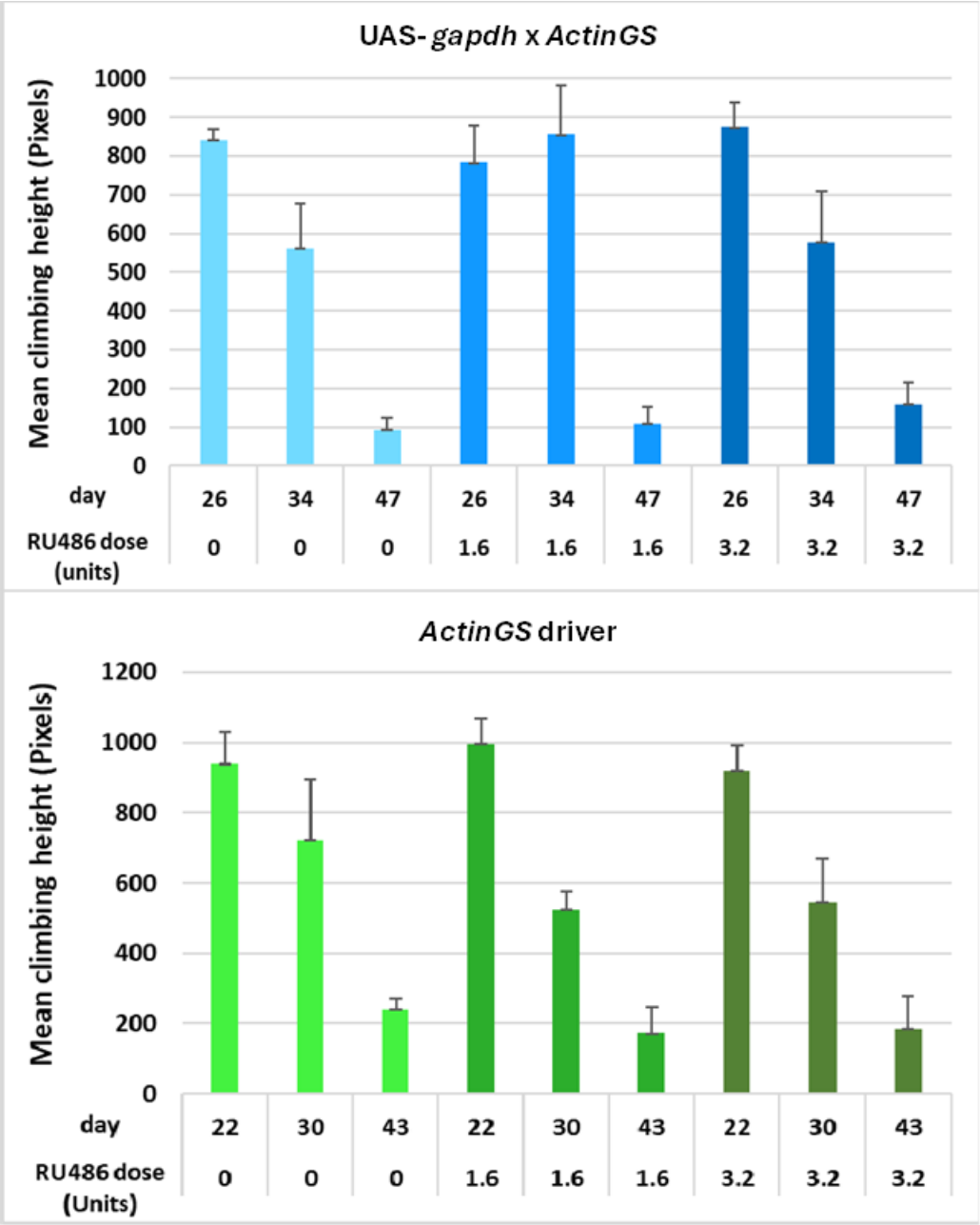

Figure 9. Mean height climbed by *ActinGS* driver vs UAS- *gapdh* x *ActinGS* flies for control flies and all Ru486 treatments. Climbing ability was evaluated via negative geotaxis at multiple time points (Days 22–47) across three RU486 doses (0, 1.6, and 3.2 µg/mL). Climbing height was measured in pixels using ImageJ analysis of standardized photographs taken 15 seconds after stimulus. Top panel showing Mean climbing height of UAS-*gapdh* x *ActinGS* experimental flies. Bottom panel showing the mean climbing height of *ActinGS* driver flies. Bars represent mean climbing height, and error bars indicate the 95% confidence interval (95% CI). Statistical significance was determined by ANOVA.

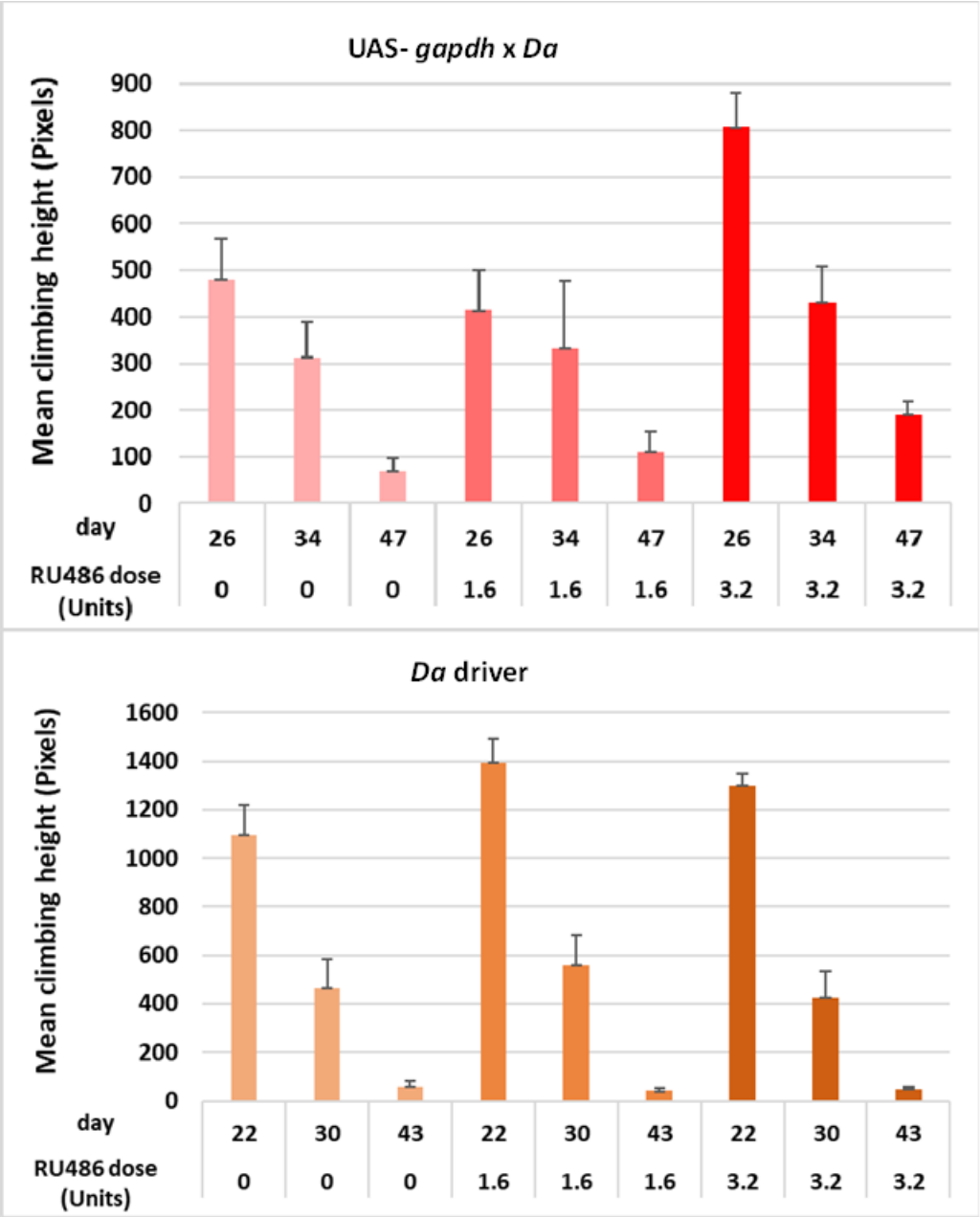

Figure 10. Mean height climbed by *Da* driver vs UAS- *gapdh* x *Da* flies for all Ru486 treatments and timings. Climbing ability was evaluated via negative geotaxis at multiple time points (Days 22–47) across three RU486 doses (0, 1.6, and 3.2 µg/mL). Climbing height was measured in pixels using ImageJ analysis of standardized photographs taken 15 seconds after stimulus. Top panel showing Mean climbing height of UAS- *gapdh* x Da flies. Bottom panel showing the mean climbing height of Da driver flies. Bars represent mean climbing height, and error bars indicate the 95% confidence interval (95% CI). Statistical significance was determined by ANOVA. A significant interaction was observed, indicating the effects of RU486 on climbing performance were dependent on genotype and age.

3.2 Investigating the effect of Antibiotic (50ug/ml chloramphenicol) on lifespan of D.melanogaster.

To investigate whether clearing bacterial infections would influence health or longevity, antibiotics (50ug/ml chloramphenicol) were administered on days 30 and 48. Figure 11 illustrates the survival plots for UAS-*gapdh* flies across both drivers. Under antibiotic treatment, UAS-*gapdh* x *Da* and UAS-*gapdh* x *Actin* flies showed median lifespans of 55 and 57 days, respectively (Table 4). While the treatment did not extend the mean lifespan of UAS-*gapdh* x *Actin* flies. Qualitatively, flies treated with antibiotics appeared healthier and performed better than untreated controls (figure 11). However, due to the late administration of the drug, negative geotaxis assays were not conducted. Early implementation of these assays might have provided clearer insights into how antibiotics influence the functional health and climbing performance of these lines.

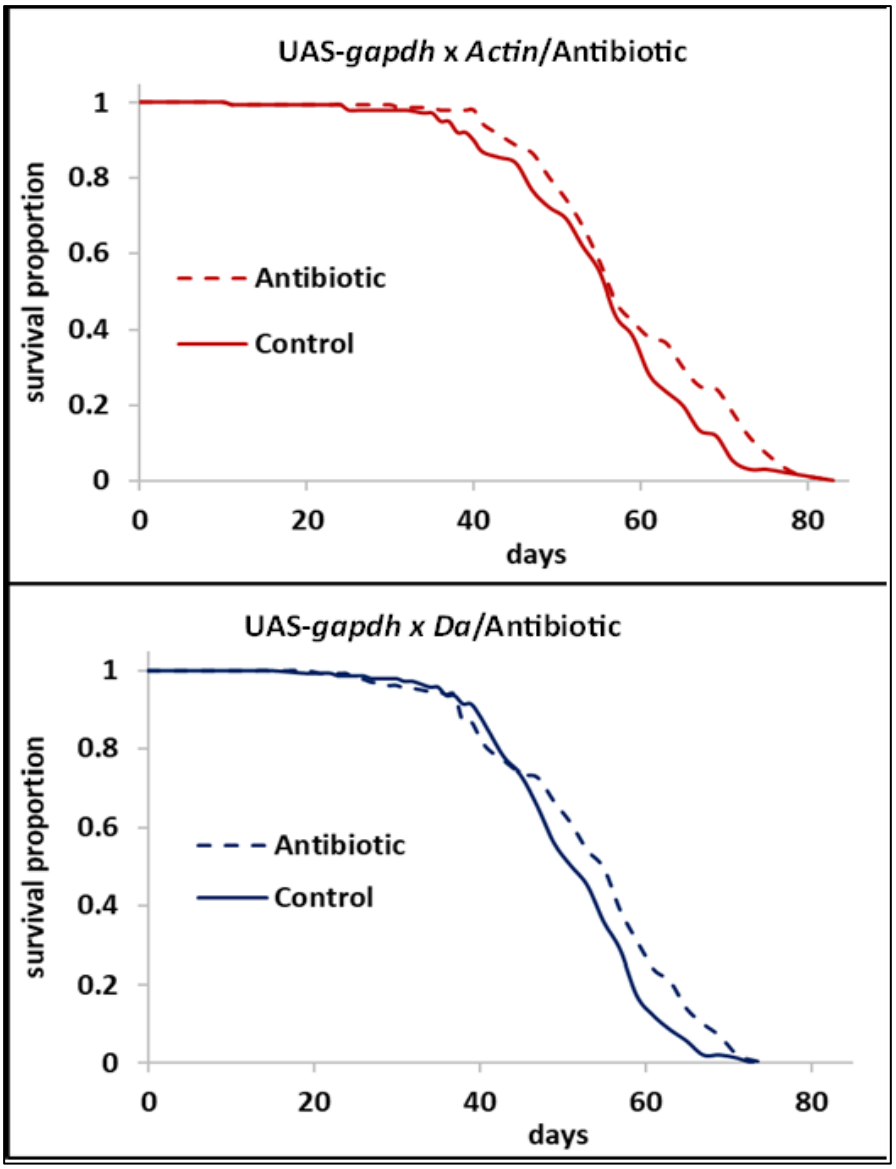

Figure 11. **Survival curves for Antibiotic (Chloramphenicol 50ug/ml) fed UAS-*gapdh* flies with both strains.** Flies were supplemented with the antibiotic Chloramphenicol (Chloramphenicol 50ug/ml) to assess the impact of microbiota suppression on the lifespan of *gapdh*-overexpressing strains. **Top panel showing** survival of UAS-*gapdh* x *Actin* flies treated with Chloramphenicol with median lifespan of 57 days, N=94. **Bottom panel shows** survival of UAS-*gapdh* x *Da* with Chloramphenicol with median lifespan of 55 days, N=84. Solid lines represent control flies, while dashed lines represent the antibiotic fed cohorts.

Table 4. Median survival data and 90% mortality for RU486 dosed flies.

RU486	**Strain**	**Median Life span**	**90% Mortality**	**Sample Size**
0	*ActinGS* driver	53	65	98
	Da driver	49	61	76
	UAS-*gapdh* x *ActinGS*	57	71	96
	UAS- *gapdh* x *Da*	51	65	85
1.6µg/mL	*ActinGS* driver	49	61	87
	Da driver	49	63	68
	UAS- *gapdh* x *ActinGS*	57	71	86
	UAS- *gapdh* x *Da*	49	67	95
3.2 µg/mL	*ActinGS* driver	43	59	85
	Da driver	47	59	88
	UAS- *gapdh* x *ActinGS*	51	66	84
	UAS- *gapdh* x *Da*	49	65	100
Chloramphenicol 50ug/ml	UAS-*gapdh*x *ActinGS*/Antibiotic	57	74	94
	UAS-*gapdh*x *Da*/Antibiotic	55	69	84

3.2.1 Expressing *gapdh* by RT-PCR

Gapdh expression levels were quantified using RT-PCR to confirm the efficacy of the GeneSwitch induction system. Due to unforeseen sample loss and experimental time constraints, expression analysis was restricted to the *Actin* driver lines; so, induction levels in the *daughterless* lines remain unverified. The analysis compared three experimental groups (n=4 per group): a low-dose RU486 treatment (1.6µg/mL), a high-dose RU486 treatment (3.2 µg/mL), and a control group (70% ethanol).

Quantitative analysis revealed a dose-dependent upward trend in expression, though neither dose achieved statistical significance. The low-dose treatment produced a 1.25-fold increase compared to the control, but the wide 95% confidence interval (0.46-2.93) and high p-value (p=0.49) show substantial variability within the samples. The high-dose treatment showed a more pronounced 1.55-fold increase with 95% confidence interval of 0.61-3.94 (p=0.193). While this result approached significance (p=0.193) more closely than the low dose, it still did not meet the p = 0.05 threshold.

These data suggest that while the UAS-*gapdh* may be responding to the RU486 inducer but still provides insufficient statistical power to confirm a significant overexpression. The overlap of the confidence intervals with the baseline (1.0) suggests that individual biological variation may be masking the average increase in expression levels.

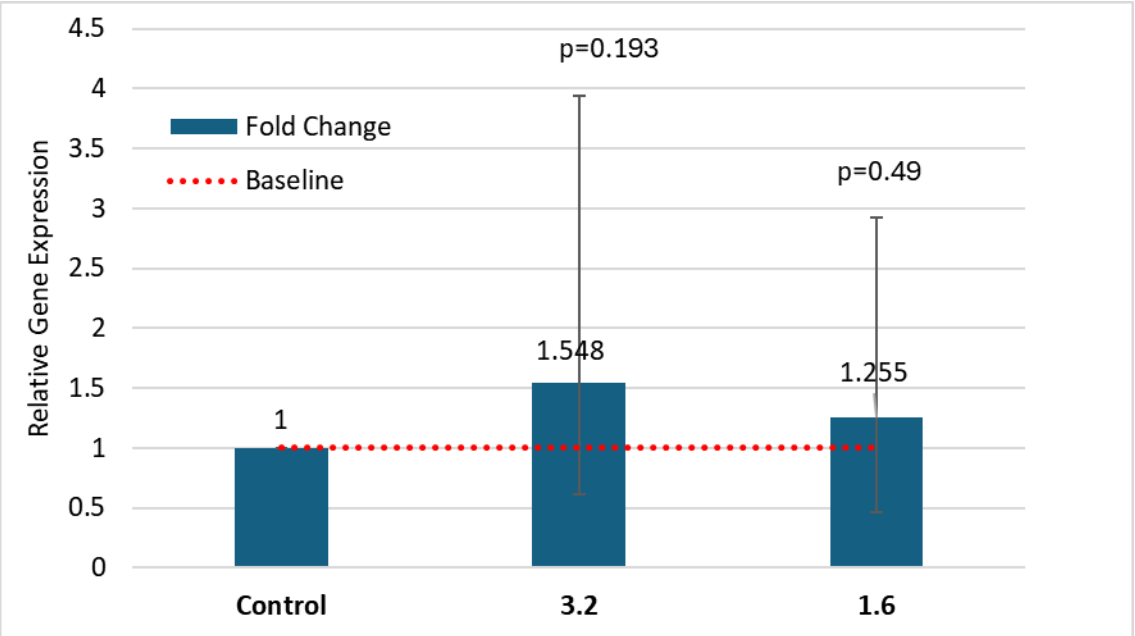

Figure 12. Relative gene expression of UAS- *gapdh* x *ActinGS* compared to control following RU486 treatment. Expression levels were quantified using RT-PCR to confirm the efficacy of the GeneSwitch system across three conditions: control (70% ethanol), low-dose RU486 1.6µg/mL), and high-dose RU486 3.2 µg/mL). Bars represent the mean fold change in expression compared to the baseline (indicated by the red dotted line) calculated via the ΔΔCT method. Mean fold changes observed were 1.255 for 1.6µg/mL dose and 1.548 for the 3.2 µg/mL dose. Error bars represent the 95% confidence interval (95% CI) derived from n=4 biological replicates per group. Statistical comparisons against the control group yielded p values of 0.49 and 0.193 for low and high doses, respectively, indicating that the increases in expression levels did not reach statistical significance ($p > 0.05$).

3.3 Effects of different drugs and their combinations in lifespan extension of D.melanogaster.

3.3.1 Survival

Earlier evidences that different drugs extended the life span of different organisms by targeting their age-related mechanisms (table 1). This project was looked to investigate that how different drugs and their combinations affected the lifespan and health span of *D.melanogaster.* Male flies from two strains, Lancaster and Dahomey (wDah) were used in this experiment. Different drugs and their combinations were applied on flies food throughout whole of their natural life span beginning on day 14 of the experiment. Four drugs were used; alagebrium (1.4mg/mL (70uL) on food), baicalein (0.2mg/mL in food), lithium chloride (15mM in food), bathophenanthroline disulfonic acid (80mg/liter in food) and NaCl (15mM) as control for LiCl.

All flies were dead on day 97. NaCl was used as control for lithium chloride and median lifespan of Lancaster flies with NaCl was 63 days. Lithium alone significantly extended the median lifespan by 6% to 67 days ($P<0.05$). And Lithium with baicalein increased the median lifespan of Lancaster flies (67 days) ($P<0.05$). Alagebrium, administered either individually or in combination with lithium, increased the median lifespan further to 69 days, representing a 3% improvement over the control (Tarone-ware p=0.001) (Table 5). While the dual combination of lithium and baicalein was beneficial (67-day median), the addition of alagebrium to this mix (the triple combination) proved antagonistic, resulting in a 6% decrease in lifespan back to 63 days ($P<0.05$). BPS alone functioned as a life-shortening agent in this strain, significantly reducing the median lifespan ($P<0.05$).

Median life span of wDah flies with alagebrium was 65 days (Figure 13). Unlike the Lancaster strain, the triple combination of alagebrium, lithium, and baicalein was highly effective for wDah, increasing the median lifespan by 3% to 69 days ($p<0.05$) (figure 13, 14). Lithium alone provided a 3% increase (69 days), suggesting that while lithium is beneficial, its impact is less dramatic in wDah than in the Lancaster strain (figure 14, 15). The specific combination of lithium, BPS, and baicalein was detrimental, pulling the median lifespan down to 65 days, which suggests a toxic interaction between these three specific drugs.

The most striking difference was the response to the alagebrium-lithium-baicalein mixture; it served as a longevity enhancer for wDah but acted as a life-shortening stressor for Lancaster flies. A commonality between both strains was the combination of alagebrium and BPS, which reduced the median lifespan by 3% to 65 days (figure 16). Baicalein, whether administered alone or in combination with alagebrium, failed to produce any significant change in the lifespan of either strain, suggesting it may not influence longevity pathways in these

specific genetic backgrounds (figure 15). While lithium generally exerted a positive effect on both strains, the magnitude of its benefit and its interaction with other compounds varied significantly between the two genetic backgrounds. Lithium chloride had a more pronounced positive effect on the Lancaster strain, increasing the median lifespan by 6% (from 63 days to 67 days, $p < 0.05$). In contrast, the benefit to the wDah strain was more modest, resulting in a 3% increase in median lifespan (to 69 days).

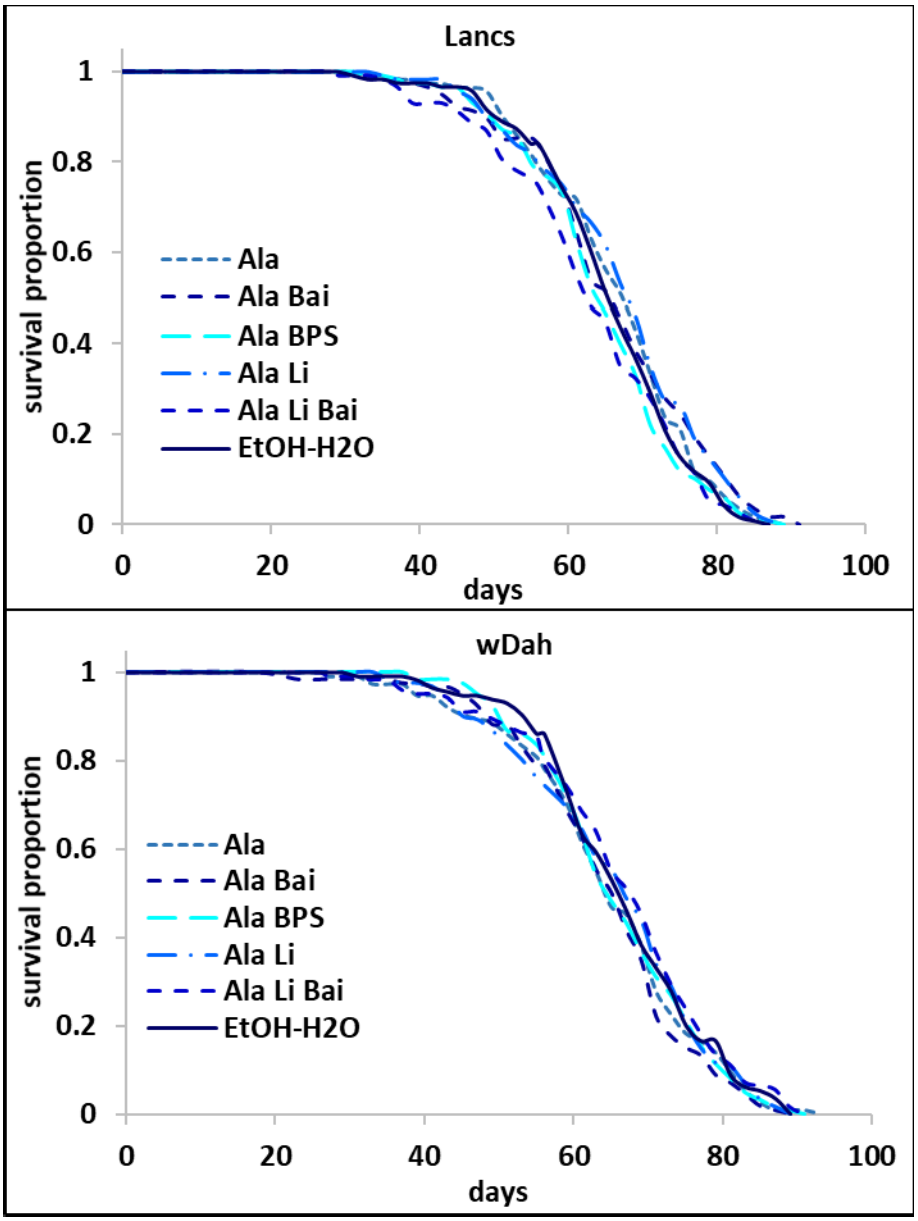

Figure 13. Survival plot of the Lancaster strain (top panel) and the wDah (white Dahomey) strain (bottom panel) under Alagebrium-based monotherapy and combination treatments. Control flies (solid dark line, Lancs, n=73; wDah, n=69). Experimental groups were administered alagebrium (1.4 mg/mL; dashed light blue Lancs, n=72; wDah, n=75), alagebrium + baicalein (0.2 mg/mL; dashed dark blue, Lancs, n=82; wDah, n=73), alagebrium + BPS (80 mg/L; solid cyan, Lancs, n=73; wDah, n=79), Alagebrium + Lithium (15 mM; dash-dot blue Lancs, n=82; wDah, n=81), and a triple combination of Alagebrium + Lithium + Baicalein (thick dashed blue line, Lancs, n=70; wDah, n=82). In the Lancaster strain, Ala significantly extended median lifespan by 3% $P<0.05$, Tarone-Ware). In the wDah strain, only the triple combination (Ala Li Bai) significantly increased median lifespan by 3% ($P<0.05$).

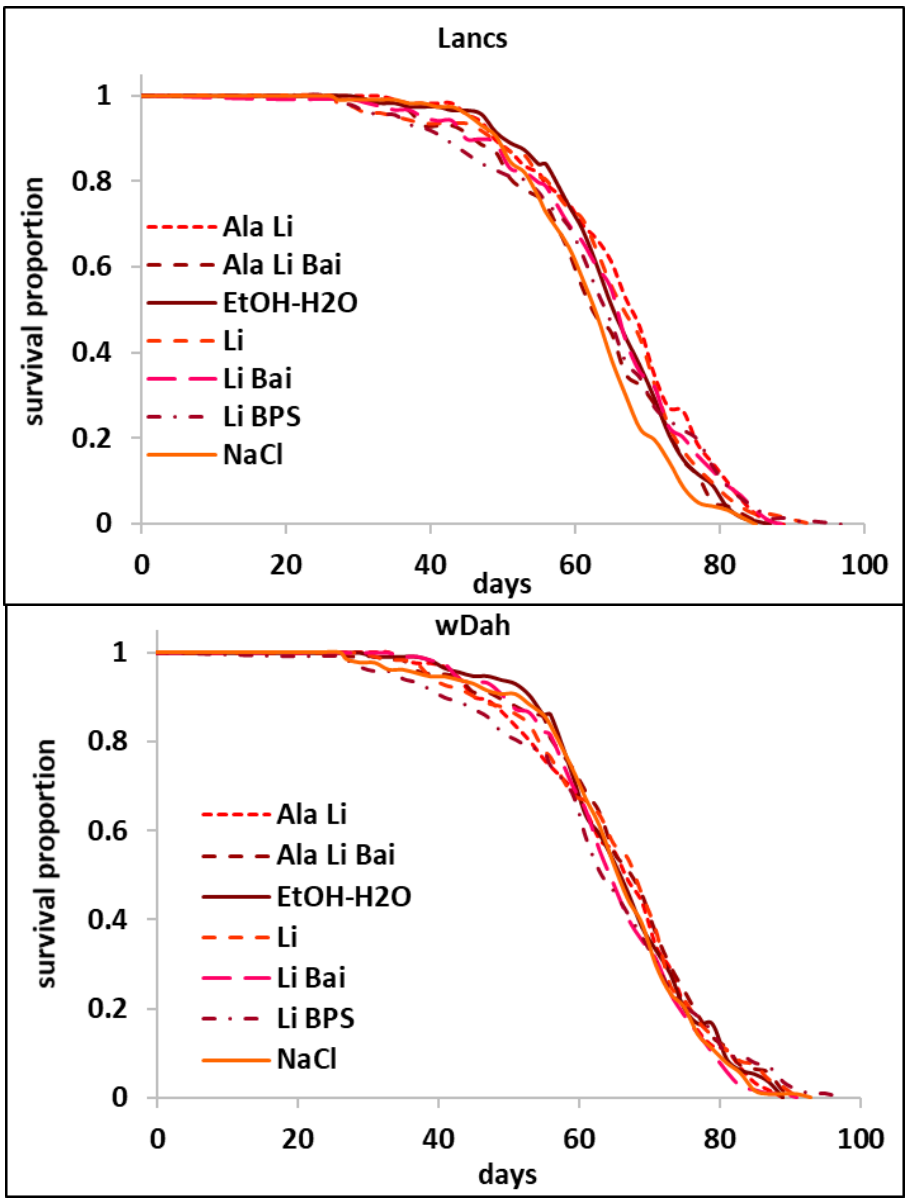

Figure 14. Survival plot of the Lancaster strain (top panel) and the wDah (white Dahomey) strain (bottom panel) under Lithium based monotherapy and combination treatments. Control flies (solid dark line, Lancs, n=73; wDah, n=69) and NaCl (solid orange line, control for lithium Lancs, n=75; wDah, n=82). Experimental groups were administered Li (15 mM Lithium, dashed orange line, Lancs, n=82; wDah, n=78), Li Bai (0.2 mg/mL Baicalein, dashed pink line, Lancs, n=77; wDah, n=76), Li BPS (80 mg/L BPS, dashed purple line, Lancs, n=89; wDah, n=85), Ala Li (1.4 mg/mL Alagebrium, + 15 mM Li, doted pink line Lancs, n=82; wDah, n=81), and the triple combination (Ala Li Bai Lancs, dashed dark line, n=70; wDah, n=82). Lithium (15 mM) significantly increased median and maximum lifespan by 6% in Lancaster and 3% in wDah strains.

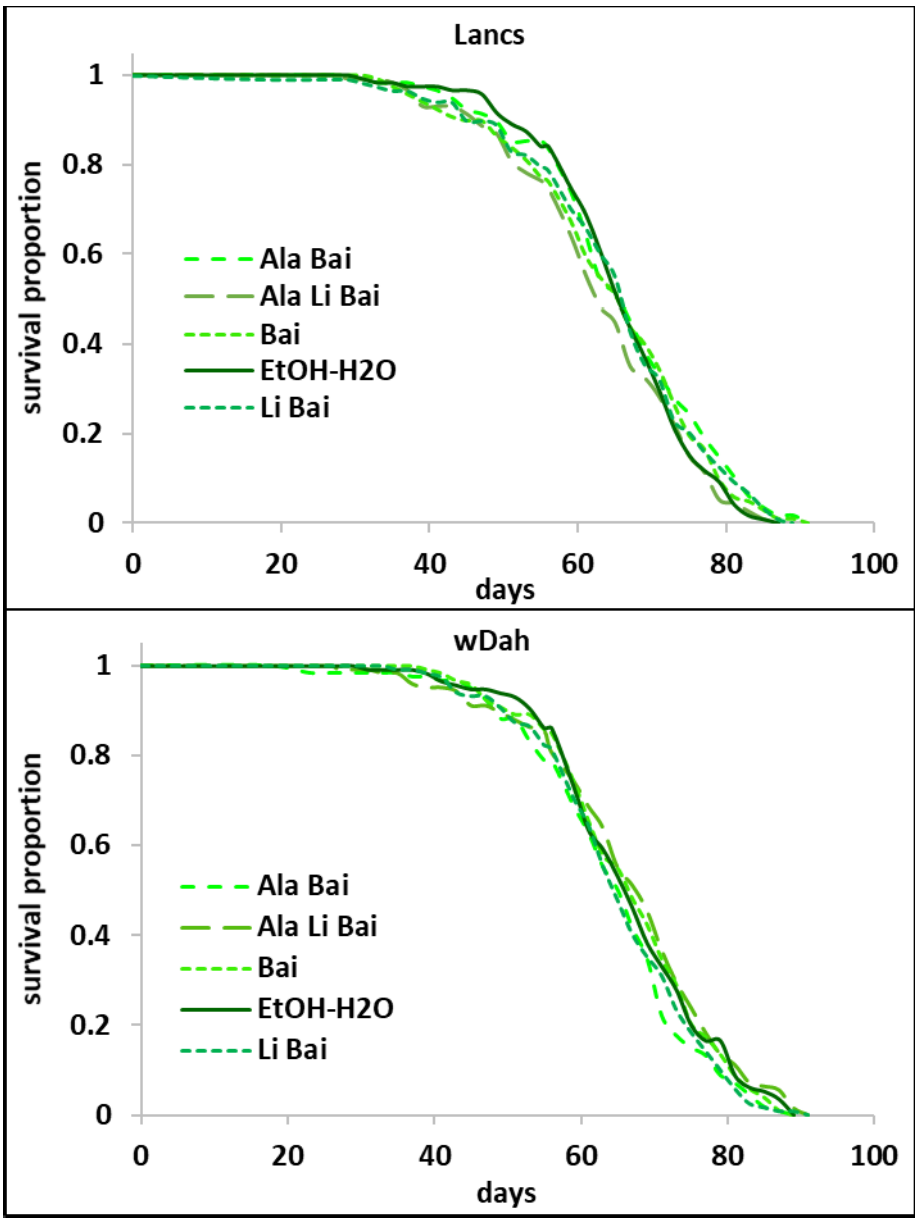

Figure 15. Survival plot of the Lancaster strain (top panel) and the wDah (white Dahomey) strain (bottom panel) under Baicalein-based monotherapy and combination treatments. Control flies (solid dark line, Lancs, n=73; wDah, n=69), experimental groups were administered **Bai** (0.2 mg/mL Baicalein, dotted light green line, Lancs, n=78; wDah, n=73), **Ala Bai** (1.4 mg/mL Ala + 0.2 mg/mL Bai, dashed light green line, Lancs, n=82; wDah, n=73), **Li Bai** (15 mM Li + 0.2 mg/mL Bai, dotted dark green line, Lancs, n=77; wDah, n=76), and the triple combination (**Ala Li Bai,** dashed dark green line, n=70; wDah, n=82). No significant effect of Baicalein alone was observed on the lifespan of either strain.

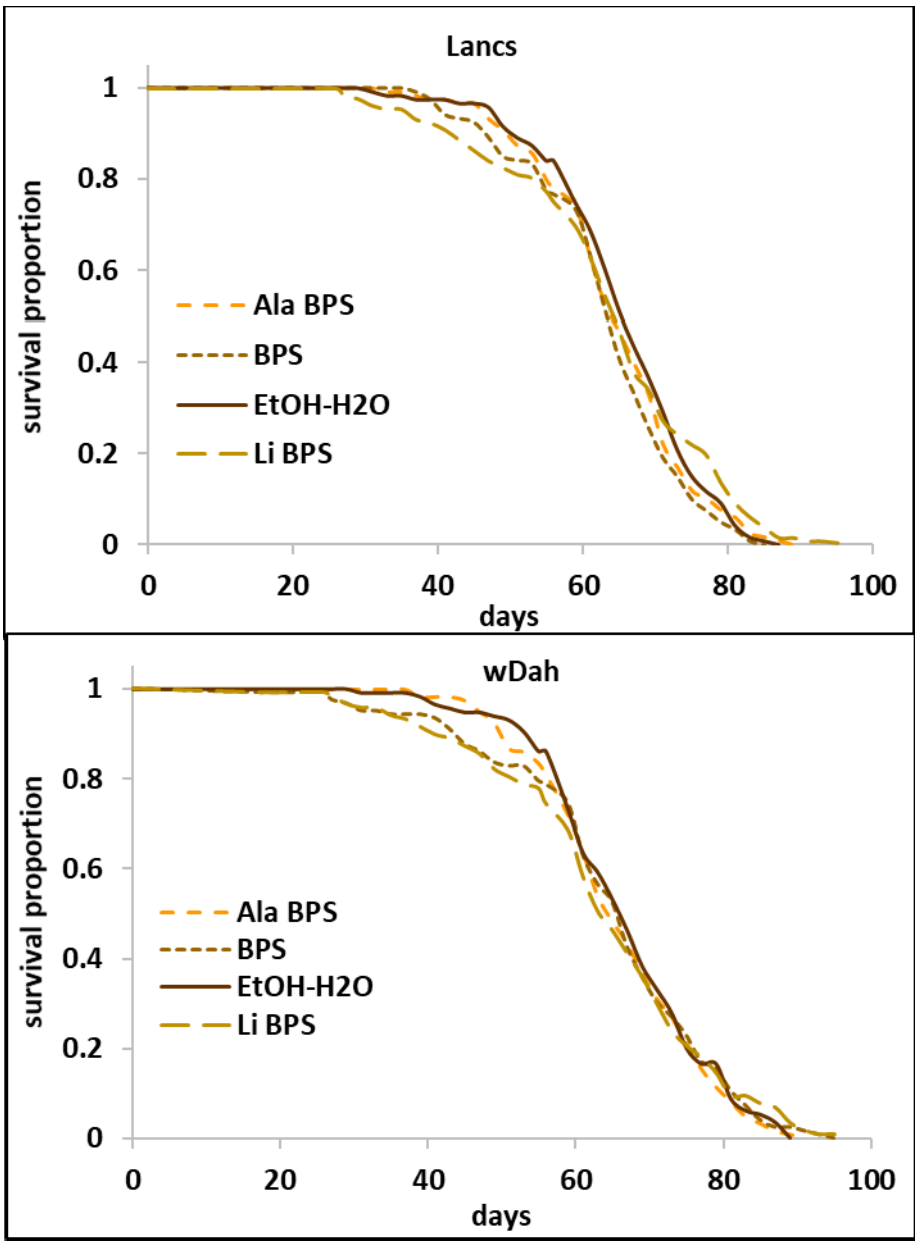

Figure 16. Survival plot of the Lancaster strain (top panel) and the wDah (white Dahomey) strain (bottom panel) under BPS based monotherapy and combination treatments. Control flies (solid dark line, Lancs, n=73; wDah, n=69), experimental groups were administered **BPS** (80 mg/L, dark dotted line, Lancs, n=74; wDah, n=85), **Ala BPS** (1.4 mg/mL alagebrium and BPS, dashed light brown line, n=73; wDah, n=79), and **Li BPS** (15 mM Li, dashed dark brown line lithium and BPS, Lancs, n=89; wDah, n=85). BPS had no significant effect on the lifespan.

Table 5. Median survival data and 90% mortality for Drug fed flies.

Drugs	Strains	Median Life span	90% Mortality	Sample Size
Alagebrium and baicalein	Lancaster	67	83	115
	wDah	67	85	117
Lithium and Baicalein	Lancaster	67	85	118
	wDah	65	85	119
Alagebrium and lithium and Baicalein	Lancaster	63	81	114
	wDah	69	85	125
Alagebrium and Lithium	Lancaster	69	83	117
	wDah	67	83	121
Alagebrium and BPS	Lancaster	65	87	118
	wDah	65	83	122
Lithium and BPS	Lancaster	65	91	132
	wDah	65	89	117
NaCl	Lancaster	63	83	117
	wDah	67	83	128
Alagebrium	Lancaster	69	87	114
	wDah	65	83	115
BPS	Lancaster	65	75	120
	wDah	67	85	122
Baicalein	Lancaster	67	83	105
	wDah	67	83	108
Lithium	Lancaster	67	91	122
	wDah	69	87	123
Ethanol	Lancaster	67	84	119
	wDah	67	85	115

3.3.2 Effects of Drugs treatment on climbing performance of *D.melanogaster* with age.

Negative geotaxis assays were conducted on day 26 of the experiment, 12 days after drug administration measured locomotor performance based on the height climbed within a 15 second interval. Negative Geotaxis was done once in the whole experiment. Overall, the Lancaster strain exhibited higher activity levels and appeared healthier than the wDah strain. However, the impact of the pharmacological treatments on climbing performance varied significantly between the two strains.

The wDah flies showed no significant improvement in climbing height with any of the drug treatments. In fact, several treatments proved detrimental. BPS significantly decreased the climbing height of wDah flies ($p<0.01$) (figure 20). Three drug combinations (alagebrium, lithium and baicalein) significantly decreased the climbing height of wDah flies (figure 17). NaCl was used as control for lithium (as mentioned above), negatively impacted the performance of the wDah flies (Figure 18). While the Lancaster flies were generally more active, they also failed to show any specific increases in climbing height across the treatments ($P=0.09$). The combination of alagebrium and lithium significantly decreased climbing height ($p < 0.05$), an effect that was much less pronounced in the wDah strain (Figure 17). Like the wDah strain, the three-drug combination (alagebrium, lithium, and baicalein) caused a significant decline in activity levels (Figure 18). In contrast to the wDah strain, the NaCl control appeared to increase the locomotor performance of the Lancaster flies (Figure 18). However, there is no specific increase in climbing height was seen in flies.

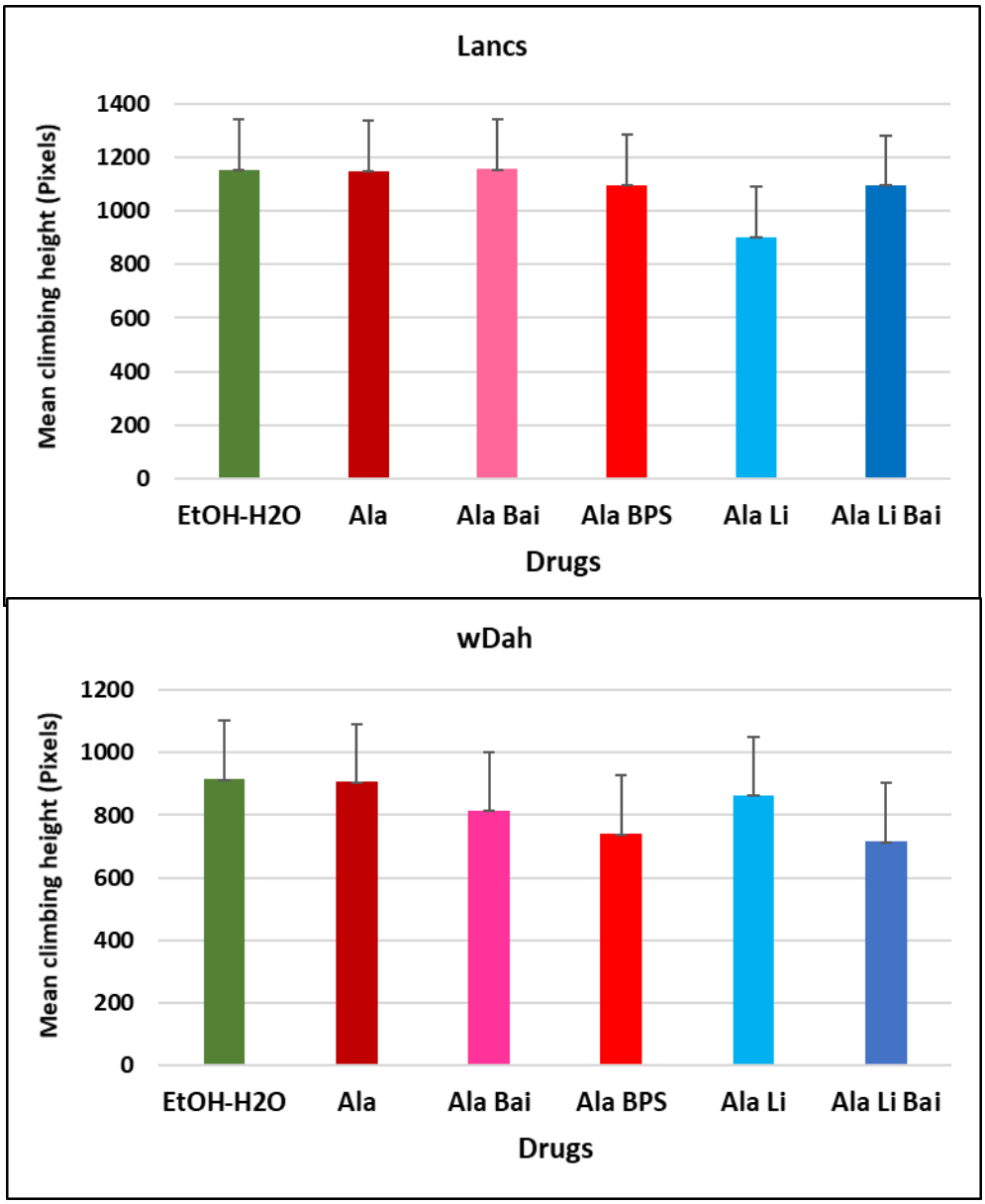

Figure 17. Mean heights walked by Lancaster and Dahomey flies from all alagebrium treatments and its combinations. Based on estimated marginal means, error bars are 95% confidence interval.

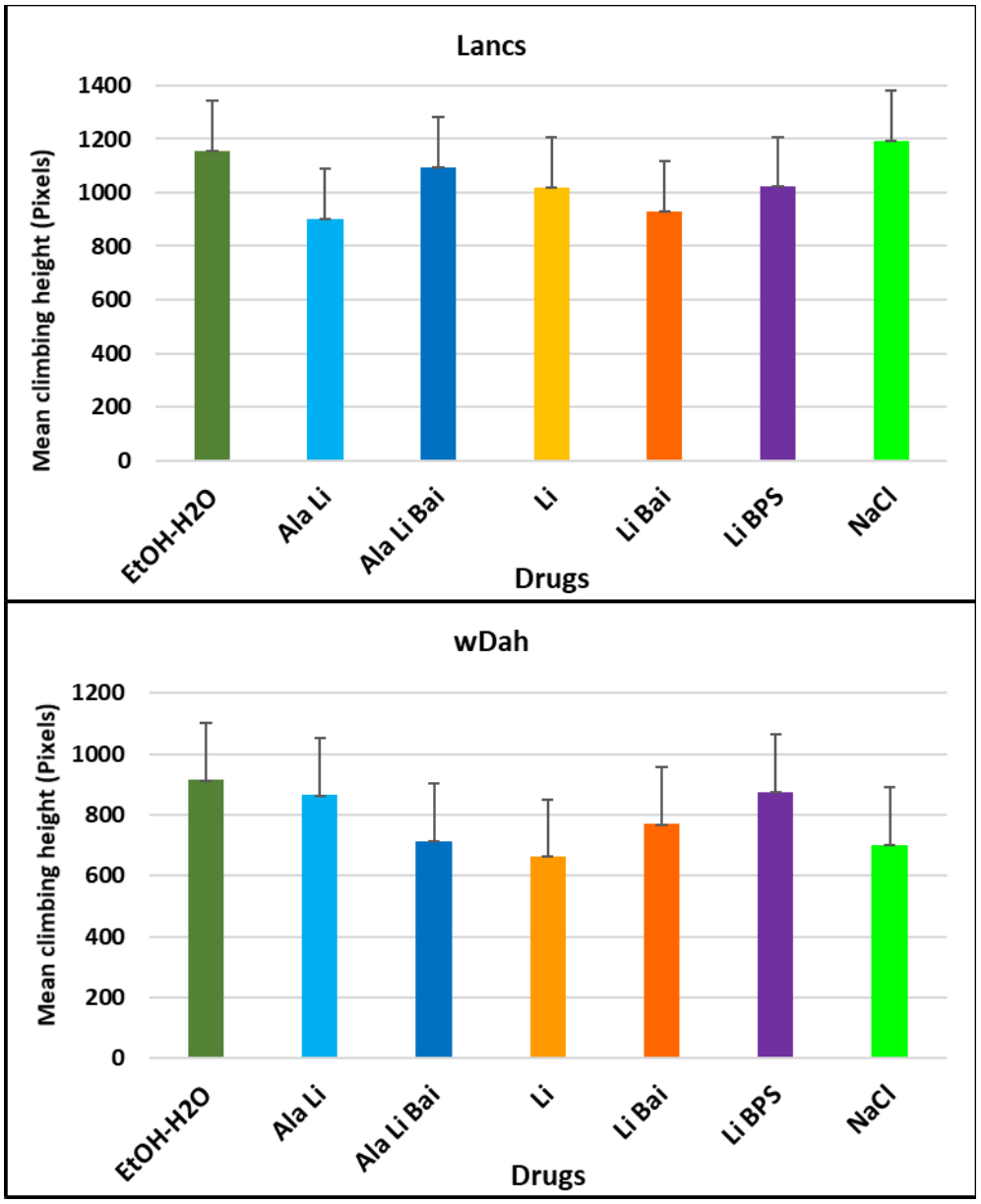

Figure 18. Mean heights walked by Lancaster and Dahomey flies from all lithium treatments and its combinations. Based on estimated marginal means where error bars are 95% confidence interval.

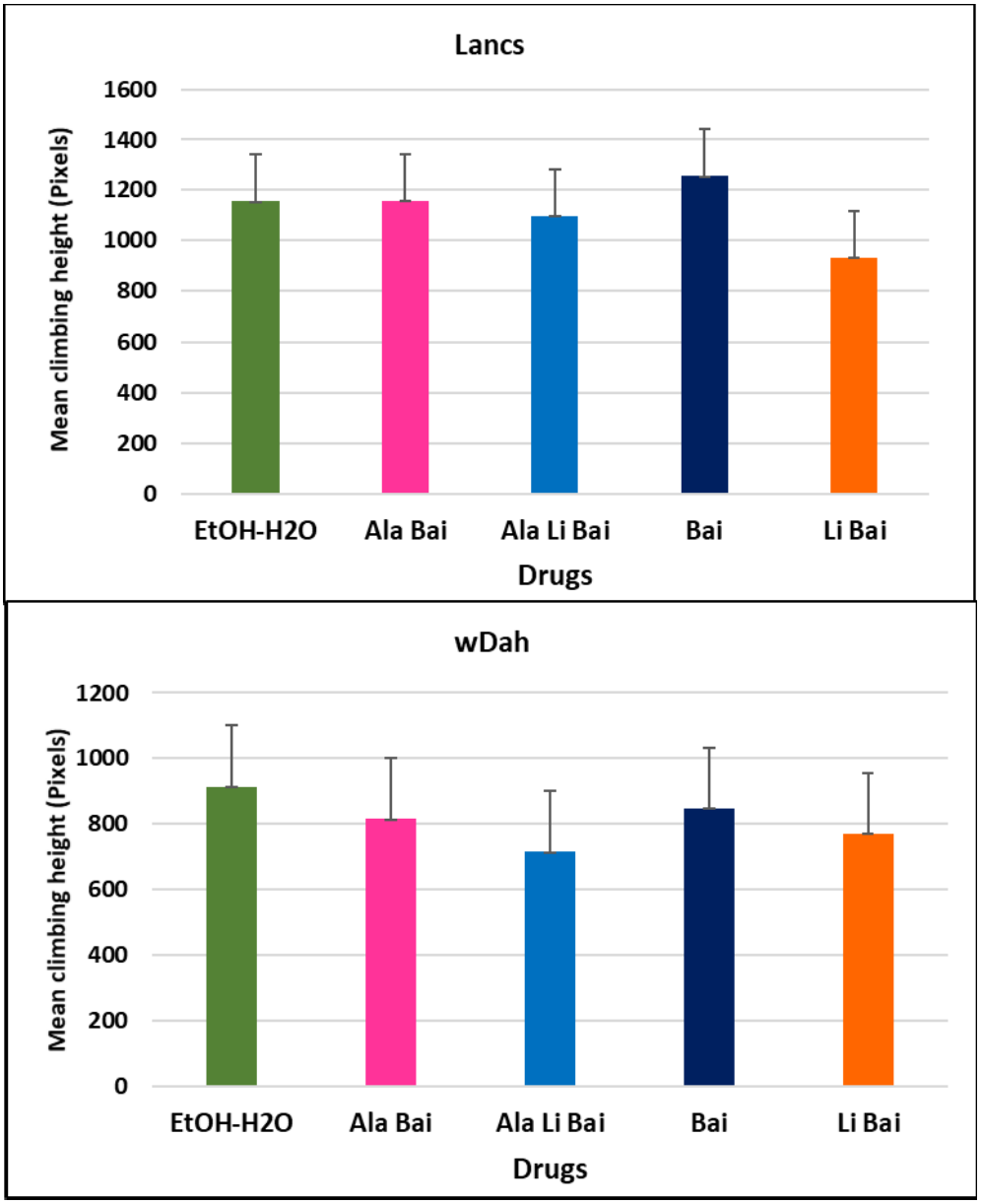

Figure 19. Mean heights walked by Lancaster and Dahomey flies from all baicalein treatments and their combinations. Based on estimated marginal means where error bars are 95% confidence interval.

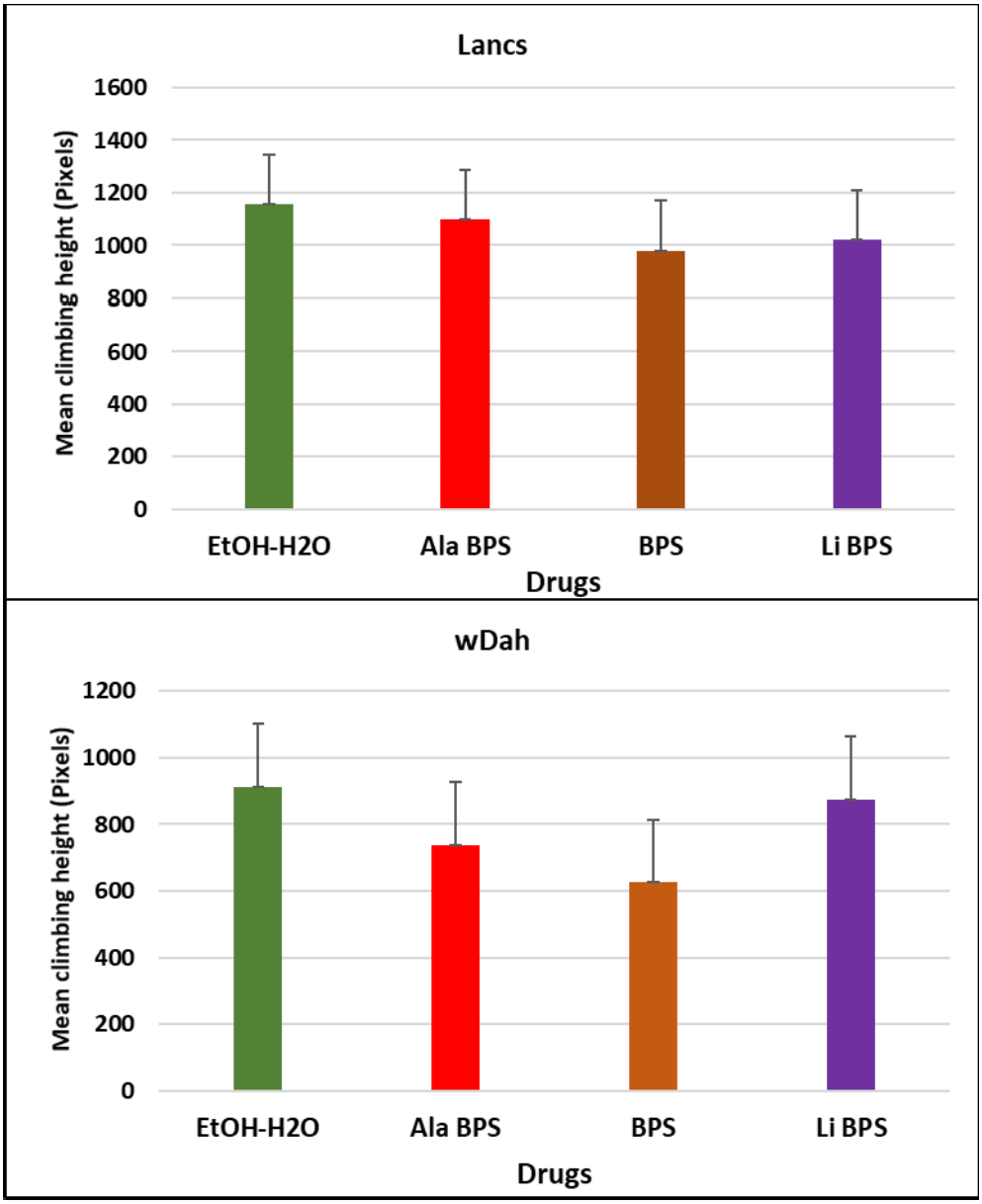

Figure 20. Mean heights walked by Lancaster and Dahomey flies from all bps treatments and their combinations. Based on estimated marginal means where error bars are 95% confidence interval.

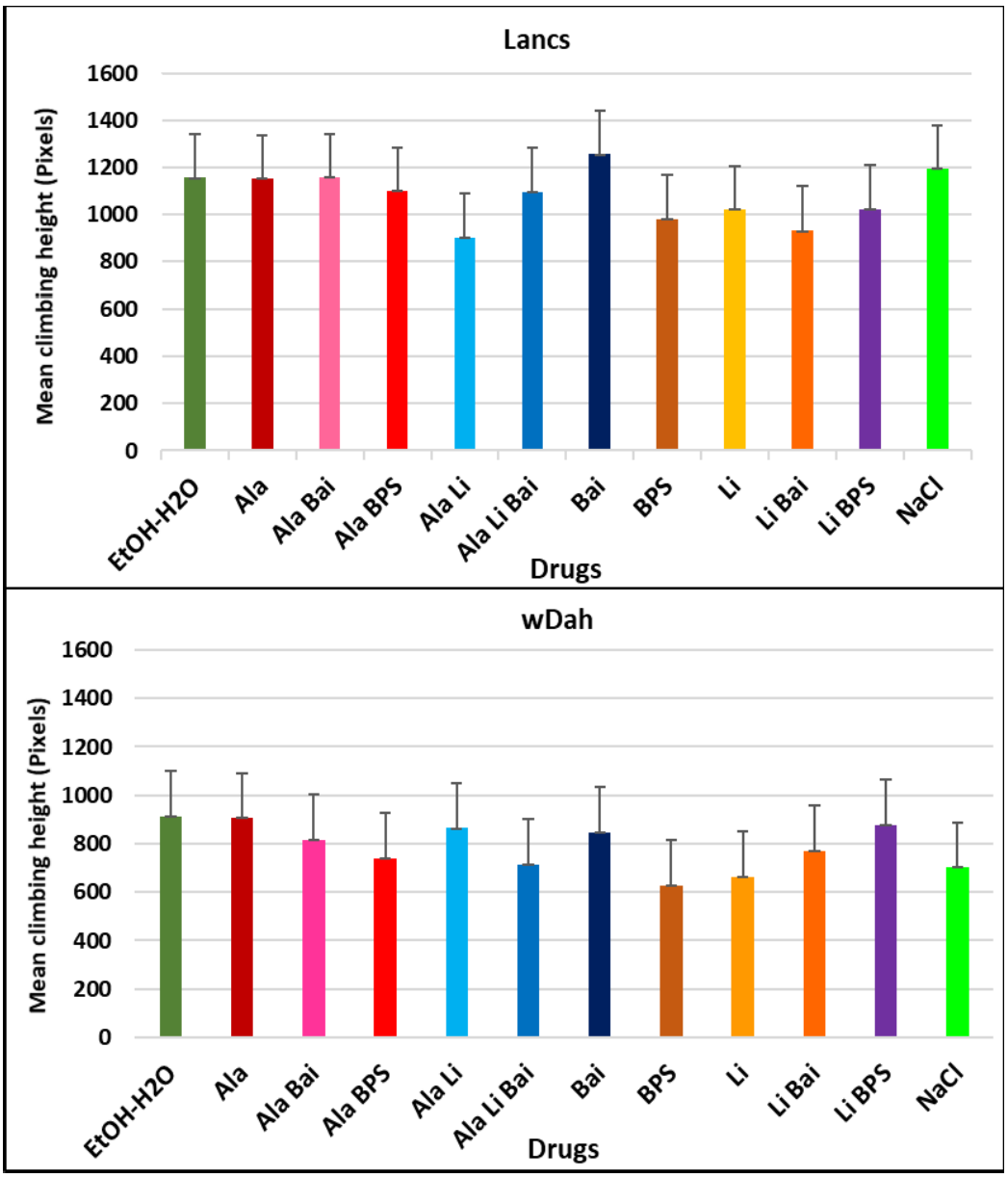

Figure 21. Mean climbing height (measured in pixels) for male Lancaster (top panel) and wDah (bottom panel) strains across all pharmacological conditions. from all drug treatments and their combinations. Data represents the mean climbing height achieved during negative geotaxis assays. Error bars (95% confidence interval) represent standard deviation. A general longitudinal decline in climbing height was observed across both strains.

4. DISCUSSION

This study investigated potential interventions to extend the lifespan and healthspan of *Drosophila melanogaster* through two distinct approaches: overexpression of the *gapdh* gene and pharmacological treatment with four different compounds, both alone and in combination.

In the first project GeneSwitch transgenic system with two ubiquitously expressing driver strains was used to overexpress *gapdh* gene during adulthood of male *D. melanogaster* flies (*ActinGS-255B* and *daughterless*) with the intension of discovering whether its overexpression could have positive effect on life and health span of flies. As well as how RU486 itself affects the lifespan of flies. Lifespan analysis and climbing performance of flies was analyzed to assess the impact of overexpression of gapdh. RT-qPCR was performed to measure the level of overexpression of *gapdh*.

The second project focused on the administration of four specific drugs, both as monotherapies and in combinations. The drugs tested were alagebrium, baicalein, lithium, and bathophenanthroline disulfonic acid (BPS). Two wild-type strains, Lancaster and Dahomey, were used to identify potential increases in life and healthspan. Monitoring for both experimental projects continued until the natural death of all subjects to ensure a complete survival profile.

4.1 *Gapdh* Experiment

4.1.1 Impact of over-expression of *gapdh* on lifespan of flies and climbing performance

Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) is commonly known for its role as a catalytic enzyme for energy metabolism, ATP and pyruvate production through anaerobic glycolysis in cytoplasm. As well as increased enzymatic activity and *gapdh* gene expression are associated with cell proliferation. *Gapdh* is involved in aging through multiple mechanisms such as *gapdh* is highly sensitive to oxidative stress because of ROS. Oxidation of its active site cysteine deactivates its enzymatic function and which in turn impairs glycolysis and cellular energy production (Tristan et al., 2011), it maintains telomere integrity by binding to telomeric DNA and protect and regulate telomerase (Sundararaj et al., 2004), *gapdh* contributes to toxic protein aggregation by binding to amyloid-β (Aβ) in Alzheimer's disease and mutant huntingtin in Huntington's disease (Ishitani et al., 1998). As well as under cellular stress, *gapdh* moves into the nucleus. There, it interacts with proteins p53 and Siah1, promoting apoptosis (programmed cell death). This pathway is implicated in processes such as aging and neurodegeneration (Hara et al., 2005). Additionally, it is also involved in many cellular processes including transcriptional and post-transcriptional gene regulation, regulation of Phosphorylation and regulating gene

transcription (Nicholls et al., 2012b). Expression of *gapdh* does not remain stable with age (Zampieri et al., 2010) but it decreases with age (Vigelso et al., 2015). Therefore, in this study, overexpression of *gapdh* was assessed on the life span as well as climbing ability of flies. Two ubiquitously expressing drivers (*ActinGS-255B* and *daughterless*) and their crosses, UAS- *gapdh* x *Actin* and UAS- *gapdh* x *Da* were used because ubiquitous expressing drivers can express in all tissues. Two doses (1.6µg/mL and 3.2 µg/mL) of RU486 were used, 70 µL of ethanol was used as control.

Lifespan was unimproved in this experiment. 3.2 µg/mL of RU486 had adverse effect on health and life span of driver flies as it decreased the median life span of *ActinGS-255B* and *daughterless* driver flies. These results are in support of previously reported that there is no increase in lifespan was seen in *Actin-255* driver strain of GeneSwitch with RU486 in both males and females, but lifespan of male flies was decreased with the RU486 (Landis et al., 2015). However, this drug was not as bad for UAS- *gapdh* x *Actin* flies however it did not increase lifespan of flies. *ActinGS-255B* flies performed better overall than *daughterless* flies. It is itself highly active and healthy strain with a good life span. The reason could be because the *Actin* strain has multiple copies of *Actin* gal 4 and so it is probably a stronger driver.

RU486 fed *daughterless* flies also had not much negative effect on them. But life span of *ActinGS* driver flies was decreased. However, it is reported that RU486 does not cause lethality, but it is possible that flies did not like its taste or did not eat food properly. As it is previously reported that the effect of RU486 on lifespan is both dose and diet dependent as On low nutrient diets, mifepristone appears to have an aversive taste, which reduces food consumption and can shorten life but drug is not detrimental on high-nutrient diets, where its effect on taste and food intake is minimal (Yamada et al., 2017). It is also reported that RU486 does not increase lifespan through a DR effect in flies, however, that the drug can have negative effects under extremely low-nutrient conditions or in other organisms like *C. elegans* (Landis et al., 2021). Quantity of RU486 given to the flies was very low (17 µL) so that flies could avoid eating the drug. Because 70 µL of ethanol was given to controlled flies and 17µL of RU486 was given to the other flies.

Negative geotaxis is a natural behavior shown by *Drosophila*, where they instinctively climb upward in response to a downward stimulus. This behavior is often assessed by tapping flies to the bottom of a cylinder and measuring how quickly they climb to the top (Gargano et al., 2005). In the present study the climbing height of flies declined with time. It was expected as it is reported previously that negative geotaxis essay decreased with age across variety of genetic background of flies (Rhodenizer et al., 2008). RU486 had negative effect on the climbing

height of *Actin* driver flies. But low dose of RU486 increased the climbing height of *daughterless* driver files. While, climbing height of flies decreased slightly as the dose was increased. These results are in favor of the previously reported data that in some cases, RU486 can cause toxicity although the dose used in this experiment was higher than used in previous finding in which RU486 was used 10mg/ml (Osterwalder et al., 2001). Low dose of RU486 increased the climbing height of UAS- *gapdh* x *Actin* flies and high dose of RU486 clearly increased the climbing height of UAS- *gapdh* x *Da* flies. But it cannot be concluded whether it is the effect of RU486 or overexpression of *gapdh*. However, it is not reported so far that RU486 has any effect on negative geotaxis of flies. Overall UAS- *gapdh* x *Actin* and UAS- *gapdh* x *Da* flies were less active than the *daughterless* and *Actin* driver flies.

RT-PCR results showed a trend toward *gapdh* upregulation, with observed increases of 1.25-fold for 1.6µg/mL and 1.548-fold for 3.2 µg/mL. However, high biological variability shown by wide 95% confidence intervals (0.46–2.93 and 0.61–3.94, respectively) resulted in non-significant p values ($p > 0.05$). Consequently, it cannot be concluded that any minor changes in lifespan were gene dependent as *gapdh* gene was not overexpressed significantly. Even though the results were not statistically significant, they are both in the expected direction, which is above 1, showing over expression. Reason that the gene was not significantly overexpressed could be some experimental errors. To avoid the experimental error such as incorrect pipetting, RT-PCR should run again in the future to get correct results. One issue here was to run RT-PCR for overexpression of the gene; flies had to be censored and frozen during the experiment. It is recommended to start the lifespan essay with a large cohort of flies so repetition of RT-PCR can be done. So, it could be reliably determined if gene is overexpressed significantly or not. The results of the present study cannot conclude that a little increase in lifespan was because of the effect of *gapdh* overexpression. Because a growing body of evidence suggests that transgenic constructs within UAS/GAL4 expression system, even in their uninduced state, can significantly increase lifespan in certain genetic backgrounds (Ren and Hughes, 2014). It is reported previously that level of gene expression in GeneSwitch system is in dose dependent manner that expression of gene can be controlled with the dose of RU486 (Osterwalder et al., 2001). And effects of RU486 itself on gene expression can mask the effect of pathway modulation. As it is described previously that quantity of RU486 given to the flies was very less, so it can be possible that flies could avoid eating the drug and *gapdh* did not expressed significantly.

4.1.2 Effect of antibiotic on life span of flies

It has been reported previously that presence of bacteria during first week of life span of *Drosophila* may increase their lifespan. However, presence of bacteria in later life decreases their lifespan (Brummel et al., 2004). In this study, flies were also fed with 50ug/ml chloramphenicol antibiotic at two time points in their total lifespan to prevent them from getting any bacterial infection to analyze if it affects the lifespan of flies. Results show that antibiotic increased the life span of UAS- *gapdh* x Da flies by 4%. It is analyzed that bacterial contamination does have effect on the lifespan of flies which suggest that part of the cause of death of flies is due to microbial effects. It is recommended that future research includes antimicrobial or antibiotic methods to achieve optimal results and the longest possible lifespan in experimental flies and also do negative geotaxis for them to ensure its effects on health of flies.

4.1.3 Future Implications

Building upon the experimental findings, this study highlights critical considerations for future investigations into the role of *gapdh* in longevity and the modification of inducible expression systems in *Drosophila*. While a possibility exists that *gapdh* overexpression could influence lifespan, the lack of statistically significant overexpression in this study precludes any definitive conclusions. Future research should prioritize confirming significant overexpression before investigating the specific mechanisms through which *gapdh* may extend lifespan. The UAS/GAL4 system remains a powerful tool for aging research due to its capacity for spatiotemporal control, though its application requires careful calibration of induction parameters. The use of the *ActinGS* driver is discouraged for future longevity studies because the significant negative impact of RU486 on the lifespan of this strain makes detecting potential extensions difficult or impossible. Future experiments should incorporate both male and female cohorts to determine if the effects of *gapdh* overexpression or drug treatments are sex specific. It is recommended to explore alternative methods for expressing *gapdh* beyond the GeneSwitch system to confirm findings across different genetic platforms. Research designs must include more robust control groups and a wider range of RU486 dosages to isolate transgene effects from inducer toxicity. Future studies must account for the context-specific side effects of RU486 (Robles-Murguia et al., 2019), such as its potential to reduce appetite and inadvertently mimic dietary restriction Landis et al., 2015). It is suggested to consider that RU486 may influence neuronal activity in clock neurons, which could alter the sleep and activity cycles of the flies (Zhao et al., 2014). Increased sample sizes are essential for maintaining the statistical power necessary to detect small but significant changes in both gene expression and survival.

4.2 Drug Treatment

Drugs and their combination can extend healthy life span when they target age-related mechanisms (Admasu et al., 2018). Based on previously published literature mention in table 1 that some drugs extended lifespan in different organisms four different drugs were used in this experiment to see if they improve life span and climbing height of *Drosophila* flies. Alagebrium (1.4mg/mL (70uL) on food), baicalein (0.2mg/mL in food), lithium (15mM in food) and bathophenanthroline disulfonic acid (BPS) (80mg/liter in food). These drugs alone and in combination were given to the male flies of two strains (Lancaster and Dahomey) to work out if they can increase life span of *Drosophila*. All these drugs were mixed in the food except alagebrium which was squirted onto the food of flies. The primary goal was to assess whether these drugs, administered both individually and in combination, could significantly improve longevity and physical performance (measured via climbing height) across different strains.

4.2.1 Effect of alagebrium

Alagebrium is known as cross link breaker formed by AGE (Advance glycation end products) (Wolffenbuttel et al., 1998). Because AGEs naturally accumulate over time (Bakris et al., 2004). Alagebrium has been studied in many diabetic animal models and has been shown to reduce diabetic complication, renal disease, diabetic cardiomyopathy and atherosclerosis (Coughlan et al., 2007). It has also been reported to improve cardiovascular function in aging rates (Candido et al., 2003). Therefore, alagebrium was used in this experiment to investigate if it can improve lifespan of flies. And results show that alagebrium extended median life span of Lancaster flies by 3% (Tarone-ware p=0.005) which is not much but it was statistically significant. In wDah flies, no lifespan extension was observed when alagebrium was administered alone. However, a triple combination of alagebrium, lithium, and baicalein significantly increased the median lifespan of wDah flies by 3%. The probable reason that it did not extend life span in wDah flies could be that dose was used this experiment might be too high or too low for the flies or that the effects differ on different genetic backgrounds. The lack of efficacy in the wDah strain when used as monotherapy may be attributed to sub-optimal dosing or strain-specific genetic variations. Nevertheless, the positive results in Lancaster flies and the successful combination therapy support the hypothesis that alagebrium holds promise for slowing age related changes (Desai et al., 2010). It would be worth repeating the current experiment with the wDah flies to see if there is any effect of this drug to increase the lifespan of flies. It is recommended for future research testing varied concentrations and administration timings to identify an optimal window for longevity. Further investigating the specific biological pathways through which alagebrium influences aging in flies.

4.2.2 Effect of Lithium

Lithium is an antiaging drug (McColl et al., 2007). As well as lithium is the only drug that is approved for human use for GSK-3 inhibition so far (Meijer et al., 2004). It has been reported that lithium chloride can increase life span of *Drosophila* for its ability to extend lifespan is primarily attributed to two pathways: by inhibiting glycogen synthase kinase- 3 (GSK-3) mechanism and activation of transcription factor NRF-2. NRF2 (Nuclear factor erythroid 2-related factor 2), transcribed from the *NFE2L2* gene, functions as a master transcription factor, fundamentally protecting cells from oxidative stress and toxic assaults. (Castillo-Quan et al., 2016). Previous research highlights lithium's efficacy, 10mM dose of lithium chloride was shown to extend the median lifespan of *C. elegans* by 46% as well as median lifespan of *C. elegans* was also extended by 46% with the 10mM dose of lithium chloride (McColl et al., 2008). In the current study, a 15mM dose of lithium significantly improved longevity in both *Drosophila* strains in Lancaster by 6% and wDah flies by 3%. These results are in support the hypothesis that lithium within the dose of 1 to 25 mM extended the lifespan of wDah flies independent of sex and genetic background. That 1mM of dose extended median lifespan of flies by 5% and maximum lifespan by 13%. As well as and 25 mM lithium increased median lifespan by 9% and 10mM dose of lithium increased maximum lifespan of female *Drosophila* flies by 4.5% (Castillo-Quan et al., 2016). Recommended dose of lithium for lifespan extension of flies should be between 0.5-25mM (Dokucu et al., 2005). However, over 50mM dose of lithium is highly toxic (Castillo-Quan et al., 2016). As it is previously reported that high dose of lithium (100mM) significantly decreased the median lifespan of *C. elegans* by 9% (McColl et al., 2008). It is recommended for future studies to use lithium within the same dose (1-25mM) for lifespan extension of flies and test for what mechanism it does affect in the flies to increase their lifespan. It also proposed a multifaceted approach for future research, including dose-response optimization, biochemical verification of GSK-3 inhibition, and metabolomic profiling to investigate the interaction between Lithium and glycolytic intermediates.

4.2.3 Effect of baicalein

Baicalein is a flavonoid compound isolated from the root of a Chinese medicinal traditional herb *Scutellaria baicalensis* which is known for its antioxidant, anti-biotic properties as well as it is a good free radical scavenger (Gao et al., 1999) and iron chelator. Research has demonstrated that the rate of age-related iron accumulation strongly correlates with the rate of physiological decline in both *Drosophila* and mice (Massie et al., 1985). And life span of *C. elegans* was extended by inhibiting iron dependent ferroptosis (Jenkins, 2020). In this study, a dose of 0.2 mg/mL was administered based on previous literature (Gao et al., 2016a) which reported

substantial increases in mean (20%) and median (26%) lifespans in male flies. However, the current experiment yielded different results. As Contrary to prior studies, baicalein showed no significant effect on the lifespan of the flies in this experiment. While no extension was observed, the drug also produced no negative or toxic effects. The lack of observed efficacy in this trial may be due to several variables, the current food formula excluded corn, which was a component in previous successful studies. This dietary difference may have altered drug absorption or interaction. As well as current study did not perform assays for antioxidant enzymes such as CAT, GSH, or GSSG, which were the markers used in previous research to explain baicalein's benefits. Notably, in present study flies lived longer as compared to the previous 2016 study as in that study all flies died at day 64 however in current experiment all flies lived until day 91 and mean lifespan with 90% mortality was 67 days which suggest that flies used in this experiment were healthy. It is recommended, in future research, to use different dose of this drug to see if it can improve life span of flies and that may have positive effect on a different strain of *Drosophila* flies and use a range of different foods. And exploring the potential of combining baicalein with other anti-aging interventions and investigating if it can also extended the lifespan of *flies* by ROS scavenging as it did in *C. elegans* by Nrf2 accumulation and by increasing ARE-dependent luciferase activity and heme-oxygenase-1 expressions in Hct116 cells (Havermann et al., 2013).

4.2.4 Effect of Bathophenanthroline disulfonic acid (BPS)

Dietary supplementation of bathophenanthroline disulfonic acid (BPS) decreased systematic iron in *Drosophila* (Yoon et al., 2017). The result of present study demonstrates that BPS did not increase the life span of both strains, but all that can be seen is significantly reduced in maximum life span of Lancaster flies by 9% (p=0.05). These results are in support of the results of previous published study that BPS did not seem to be beneficial on normal flies and slightly decreased their life span. Moreover, high dose of BPS can be harmful to even normal flies (Wu et al., 2017). As well as 0.2mM BPS decreases the mean lifespan of *Drosophila* flies by 25% (Missirlis et al., 2006). BPS was not beneficial for lifespan extension of flies, either alone or in combination of other drugs. It can also be a possibility that dose of BPS could be too high or too low. It is recommended to try different doses with different dose timings for future for life span extension of flies. as well as measure the iron levels of the flies to see if it affects the iron levels of flies.

4.2.5 Impact of drugs on climbing height of flies

Negative geotaxis was also performed in the experiment to analyze climbing height of drug fed flies. For all treatments with all drugs, there was significant decrease in height climbed

by flies over time, as expected. There was no positive effect of drugs seen on the climbing height of flies. Decrease in climbing height with time is expected and natural as it is reported previously that climbing height of flies decrease over the time across variety of genetic background (Rhodenizer et al., 2008). Negative geotaxis was done only once in the whole experiment; it would be worth in the future to do negative geotaxis a couple of times to have a better view of the effects of drugs on the health of flies.

4.2.6 Effect of combination of drugs and future implications

The primary objective of this study was to determine if specific drugs or their combinations could extend the lifespan of *Drosophila*. The overarching conclusion is that drug combinations provided no additional lifespan extension beyond the effects of lithium alone. As Lithium Chloride (LiCl) administered alone provided a significant extension of median lifespan in both *Lancaster* and *wDah* strains. While certain combinations yielded modest increases, no synergistic breakthrough was observed. Surprising effect of three drugs (alagebrium, lithium and baicalein) combination was seen that this combination significantly increased the median life span of wDah flies by 3% which was 69 days (p=0.05). The results align with the literature suggest that drug combination can influence longevity (Admasu et al., 2018). However, baicalein itself had no effect on the lifespan of flies. Lithium itself and with combination of alagebrium did increase the median life span of Lancaster flies but this drug did not do well with baicalein combination. There is evidence that combinations may reduce individual drug side effects (Levine and Cagan, 2016) for example, the Lithium and BPS combination increased maximum lifespan in both strains, even though BPS alone reduced the lifespan of Lancaster flies. As well as BPS in combination of alagebrium did not have any effect on file's life span but slightly reduced the maximum life span of Lancaster flies.

A critical observation was that while some treatments extended lifespan, they did not necessarily improve healthspan. The climbing height of the flies decreased over time across all groups, suggesting a decline in physical vigor. According to (Gems, 2014), a compound is classified as anti-aging, if it acts on the aging process to increase lifespan, even if it does not improve healthspan. However, But the potential benefits of drugs that target both healthspan and lifespan make them a promising area of research for addressing the growing burden of age-related diseases and likely to be beneficial for public health (Castillo-Quan et al., 2015).

To build upon these insights, future studies should assess a wider, more carefully calibrated range of doses of some drugs which were not being able to extend the lifespan as well as use various strains to identify optimal life-extending concentrations. It is crucial to perform

assays to confirm that the drugs are, in fact, inducing their presumed physiological changes in the fly model. As well as to find out the mechanism by which drugs extended the lifespan. Moreover, further experiment can be done whether these drugs achieved that physiological change which they supposed to do then we could say that these drugs do something related to the ageing. Further research is needed to explore the potential of different antiaging drugs and combining drugs with other antiaging interventions as well as investigate the efficacy of these drugs in extending lifespan and the health span in mammals. But some other things should be taken into consideration that combining drugs can be complex and requires a careful consideration of drug dosages and timing. And combining drugs can be expensive as compared to using a single drug.

4.2.7 Conclusion

The research across both projects highlights the significant complexities involved in lifespan extension studies, emphasizing that genetic intervention and pharmacological treatments do not always yield uniform or purely beneficial results.

The first experiment provided inconclusive data regarding whether *gapdh* overexpression extends lifespan. Two primary factors hindered definitive results: first was that the level of gene overexpression achieved was statistically insufficient. And the other was that significant confounding effects were observed from RU486 toxicity, which interfered with the data. The results suggest that the *ActinGS-255B* driver should be avoided in future studies due to its high sensitivity to the negative effects of RU486. The observed positive effects on climbing in the UAS- *gapdh* x *Da* cross assure further investigation to disentangle the effects of *gapdh* from the effects of the GeneSwitch components.

The second project confirmed that pharmacological manipulation of aging is highly dependent on both drug formulation and genetic background. While Lithium and Alagebrium showed evidence of lifespan extension (e.g., 6% in Lancaster with Lithium alone), these benefits were often accompanied by a decline in healthspan (reduced climbing ability), suggesting an increased quantity of life but a reduced quality of life. Effects were highly strain specific. A combination that benefited wDah lifespan (the three-drug mix) was detrimental to Lancaster lifespan, reinforcing the necessity of testing potential geroprotectors on multiple genotypes. The results suggest strong antagonistic interactions when drugs are combined (e.g., the three-drug mix reducing lifespan in Lancaster), showing that combining single acting geroprotectors does not guarantee synergistic benefits. Future research should focus on optimizing the concentration of promising drugs like Lithium while rigorously monitoring the associated functional decline to achieve a simultaneous extension of both lifespan and healthspan.

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