Evolutionary and plastic responses of *Drosophila* under predation risk

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A Thesis Submitted to the School of Graduate Studies in the Partial Fulfillment of the Requirements for the Degree M.Sc. Thesis

McMaster University

M.Sc. Thesis (2017)

Hamilton, Ontario (Department of Biology)

TITLE: Evolutionary and plastic responses of *Drosophila* under predation risk

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NUMBER OF PAGES: xi, 127

Lay Abstract

Predation is a profound selective force, with many anti-predator adaptations seen throughout the diversity of life. Antipredatory behavioural adaptations must balance immediate and future fitness effects, to maximize overall fitness. In *Drosophila melanogaster*, research into natural ecology and role of predation is generally lacking for behavioural & evolutionary studies. I will discuss research on the influence of predator exposure on *Drosophila* behaviours, as well as the evolution of *Drosophila* behaviours and genomes through experimentally evolved populations of *Drosophila*. While predation risk has resulted in changes in *Drosophila* locomotory activity, predation has not altered mating behaviours.

Abstract

The "risk allocation hypothesis" can predict how prey species will respond to predation risk, balancing vigilance with potentially risky behaviours. In order to maximize fitness, an optimal behavioural repertoire can evolve to respond to predation threat to allow for both survival and future fitness gains. High locomotor activity and time spent engaging in mating behaviours are expected to put *Drosophila melanogaster* at a greater risk to predation. With direct predator exposure, *Drosophila* are predicted to reduce activity and mating, which over years of exposure, will be reflected in evolved behavioural traits and evolved changes in the genome. Predation as a selective force shows alterations in flies genomes of experimentally evolved populations. Locomotor activity was found to be reduced in the presence of zebra jumping spiders (*Salticus scenicus*), presumably due to these spiders as visual hunters, using movement to detect prey. This behaviour is reflected in populations of *Drosophila* that have been constantly under selection by predators. Flies evolved with spider predators or mantid predators (*Tenodera aridifolia sinensis*) showed reduced locomotor activity when no predators are present. Interestingly, while alterations are seen for locomotory activity, the presumed risky behaviours of courtship and mating did not show an evolved response. Wild caught populations under threat from spiders, as well as the evolved populations when no predators were present showed no alterations in courtship or copulation behaviour. It appears that although there may be potential risks associated with mating behaviours, the benefits to future fitness when mating outweigh the potential costs from predation risks in *Drosophila*.

Foreword

This thesis covers my work through two years of work, with focus on the plastic and evolutionary response to predation in *Drosophila* behaviours and genetics. I will discuss results from many experiments in two chapters, with chapter "1" covering experiments looking at the behaviours of *Drosophila melanogaster* both with direct predator cues and over evolutionary time with experimentally evolved populations within the laboratory. Chapter "2" will cover the genomic evolution of *Drosophila* with populations of experimentally evolved populations with high predation risk, expecting the populations to show genomic shifts due to predation selection to higher fitness peaks. Each chapter is presented separately, and may have overlap with some ideas and I apologize for any redundant information that appears to be presented twice. However, this layout allows for a clear separation in the behavioural evolution vs. the genomic evolution of *Drosophila*, which allows each section to focus primarily on the traits of interest.

The data presented was a collaborative effort with many people contributing to the work. Although acknowledged previously, the data collection by members of the Dukas lab (Dr. Reuven Dukas and Erik Etzler) and members of the Dworkin lab (Abhijna Parigi, Dr. Michael DeNieu and Mauricio Losilla) was an invaluable asset and contributed to greatly to my research.

Acknowledgements

First and foremost, thanks to my supervisor, Ian (Dr. Dworkin to some) for taking me on and helping me through experimentation and frustrating data analysis, and for teaching me many skills and tricks of the trade that have got me to where I am today. Thanks to all members of the Dworkin lab, past and present for help that is to much to include here. Also thanks to members of the Dukas lab for allowing me to use some experimental materials, lab space and help with many experiments. Many individuals contributed with data collection that deserve tremendous thanks, specifically Dr. Reuven Dukas and Erik Etzler for collection of locomotor activity data and evolved courtship in light vs. dark, Abhijna Parigi for the mating experiments with evolved populations at different ages, and Dr. Michael DeNieu and Mauricio Losilla for work with the evolved populations, specifically selection of the episodic predation population, along with DNA extractions/ sequencing set up. Also thanks to Dr. Brian Golding for allowing me access to his server to run the large data sets, as well as helping install packages and software necessary to my analysis. I also need to thank Miroslav Cika from McMaster for building the assay vials for the avoidance experiments. Thanks to my committee members (Dr. Dworkin, Dr. Dukas and Dr. Evans) for meeting with me and all the positive feedback I have received. Lastly, thanks to Melissa (if you ever read this) for putting up with my late nights working and rambling explanations of my experiments and data.

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Chapter 1

Natural and Evolved Behavioural Response to Predation

1.1 Introduction

1.1.1 General Introduction

Predation may be one of the most influential selection pressures throughout evolutionary time, resulting in a diversity of adaptations in morphology, behaviour and performance (Bengtson 2002). While predator induced natural selection can result in adaptations increasing the odds for survival for prey, the predators can also undergo selection matching these changing prey, resulting in predator-prey arms races (Dawkins and Krebs 1979, Dietl and Kelley, 2002). A wide array of antipredatory adaptations have been observed, such as defensive structures (Hoso and Hori 2008, Palmer 1985), camouflage techniques (Stuart-Fox *et al.* 2008) or poisons and warning colourations (Myers *et al.* 1978, Williams *et al.* 2012), as

a result of predators as agents of natural selection. These may in turn promote the evolution of predators to bypass these defenses, such as snake species gaining resistance to the chemical defenses of newts (Williams *et al.* 2012), or frogs (Myers *et al.* 1978). This red-queen arms race is in constant flux to match the ever changing adaptations of selective counterparts, in order to increase the fitness of either species (Dieckmann *et al.* 1995).

Generally fitness is partitioned into three components: viability, fecundity and mating success (Lind and Cresswell 2005, Orr 2009), which can be generalized into immediate fitness and future fitness. Immediate fitness will be an individual's ability to survive a potentially lethal event, while future fitness includes resource acquisition, such as mating, territory or foraging opportunities. Within predator-prey interactions, predators are focused on their future fitness while the prey are focused on their immediate survival. However, focus on immediate fitness may result is conflicts, leading to a potential loss for future fitness opportunities, as devoting energy to one fitness aspect lowers investment to other fitness effects (Creel and Christianson 2008). Some beneficial adaptations for survival may be detrimental to other future fitness effects, which in the end can decrease overall fitness. Evolution therefore may be expected to balance immediate survival and future fitness opportunities (Andersson 1994, Orr 2009).

There are many examples of antipredatory adaptations to increase survivorship. However some traits - often a result of various forms of sexual selection (Andersson 1994, Darwin 1871) - can have negative impacts on survival. Sexually selected traits can increase predator detection or capture, including bright colourations (house finches; Hill 1990, guppies; Kodrick-Brown 1985), large ornamentations

(long-tailed widows; Andersson 1982, stalk-eyed flies; Cotton *et al.* 2004), or courtship calls (field crickets; Wagner and Reiser 2000, wolf spiders; Kotiaho *et al.* 1998). Although these traits may lower viability, they persist in many species (Candolin 1998, Lima and Dill 1990, Magnhagen 1991). Despite the potential increased risk of capture (viability selection), evolution favours these adaptations as they have sufficiently large impacts with respect to mate acquisition (sexual selection), and the benefits to this fitness component outweighs the potential costs (Orr 2009).

Despite the high potential cost, predation risk varies spatially and temporally for most organisms, which may result in the evolution of different behavioural strategies utilized by prey depending on the nature or likelihood of a predator encounter. This context dependent display of behaviours (plasticity, Mery and Burns 2010) can modulate risk in predator encounters, such as with the wolf spider (*Hygrolycosa rubrofasciata*), able to modulate mate searching and courtship drumming based on the perceived risk (Kotiaho *et al.* 1998). Although these spiders have to opportunity to engage in behaviours that would be beneficial for mate acquisition, under predation risk these behaviours are suppressed in order to increase the probability of survival. Similarly to morphological adaptations, there will be optimal behavioural phenotype for prey to maximize overall fitness. However optimum is not simply just the trait mean (or activity level of a particular behaviour), but in context dependent use of behaviours. This balance is needed, as behaviours beneficial for future fitness effects (i.e. access to mates, foraging opportunities etc.) may be lost when engaging in antipredatory behaviours, a loss representing a type of non-consumptive effect (Creel and Christianson 2008, Lima

1998).

Consumption by predators is the obvious direct cost to prey with the loss of opportunity to increase any future fitness beyond the death of the individual. However, there are also non-consumptive effects on fitness that an individual may experience. These effects include energy and resources lost due to indirect, non-lethal interactions with predators, such as time spent expressing vigilant behaviours, or fear of predators resulting in potentially lost opportunities for increasing future fitness (Creel *et al.* 2014, Lima 1998, Preisser and Bolnick 2008). These losses of future fitness effects can be in the form of altered prey group sizes, the loss of foraging opportunities or effective mating opportunities (Creel and Christianson 2008, Creel and Winnie 2005, Sih *et al.* 1990). For prey there is potential costs that must be weighed when encountering a predator. Increasing investment in behaviours beneficial for survival can result in lost foraging/mating opportunities, while engaging in these foraging/mating behaviours may increase the odds of predator capture.

Generally the simplest manner to lessen the fitness costs of predation (both consumptive and non-consumptive) is to avoid any encounters with predators (Lima and Dill 1990). Actively engaging in potentially risky behaviours during times and locations known to be without predators results in little predation risk to immediate survival. One theory for the evolution of nocturnal animals was the selective advantage of engaging in night time behaviours due to less active predators at night (Gerkema *et al.* 2013, Kotler *et al.* 2010, Walls 1942). When a predator does enter the vicinity of a prey, early detection by prey will allow for continuing avoidance of predators all together, which limits predator interactions. This

will lessen consumptive (capture and death) effects associated with predation risk. However avoiding the locations with the predators may limit resource acquisition at multiple levels. Behaviourally, we can see adaptations that can increase an individual's survival, most obviously head raising while foraging in many species, which is able to be decreased with aggregation into groups (Bednekoff and Lima 1999). Some species instead rely on one sentry to be a lookout for predators while others are able to forage, as seen in meerkats (Clutton-Brock *et al.* 1999). Even though this sentry is losing foraging opportunities, it is believed they have a greater advantage to survive as they will be those with initial detection and most time to escape to safety (Bednekoff 1997, Clutton-Brock *et al.* 1999). The ability to efficiently detect predators early and avoid times and locations of high predation risk can limit non-consumptive effects and increase the immediate fitness benefits of prey

However, if avoiding predators imposes a great cost to the individual prey in the future (i.e. extended hiding causing lost foraging opportunity), it may be more beneficial to forgo avoidance, despite an increased risk of predator capture. As there is a chance predators may ignore the prey when not actively foraging or other prey species within the area are of interest to predators, prey may chance attempts to lessen the non-consumptive effects due to high predation risk. However, if predators are hunting and aim to capture exposed prey, apart from the obvious costs of injury or death, those that escape may experience a detrimental fitness cost due to a large expenditure of energy escaping. This can lower future fitness, such that the behavioural decision to engage in risky behaviours must be worth all the potential costs associated with these behaviours. It is the relative detrimental

effects on the prey's future fitness that will determine when it is more beneficial to engage in these increased risky behaviours.

The effects of predation risk on future fitness is believed to be determined by the levels of risk associated with predator-prey interactions, and how much time an individual spends in high risk environments. This results in changes in the allocation of vigilance behaviours compared to resource acquisition at times of high predation risk, known as the "risk allocation hypothesis" (Lima and Bednekoff 1999). If the time at high risk is short and infrequent, engaging in vigilance behaviours will be beneficial during the times of high risk. As the time spent investing in high vigilance behaviours is short, there is little accumulation of the non-consumptive effects, which can be compensated with longer proportions of time at low predation risk. However, with persistent, more frequent and longer bouts of predation, the costs of focusing all energy to immediate survival (via vigilance etc.) can have detrimental effects further in the prey's lifetime, as the opportunities for resource acquisition is reduced. There is an accumulation of non-consumptive effects with high risk and high vigilance, both due to the time at high risk, and a reduced time at low risk to compensate for the lost opportunities due to predation. This model (Lima and Bednekoff 1999) predicts that high risk can lead to a reduction of antipredatory behaviours (vigilance) in favour of resource acquisition (foraging, mating etc.).

The empirical support for this model to accurately predict prey responses has been mixed, in particular through different experiments looking at the learned response to variable predation risk (review by Ferrari *et al.* 2009), but observation

of natural populations with varying predation risk generally show support for responses based on the "risk allocation hypothesis" (Creel *et al.* 2008, Kotler *et al.* 2008, Zanette *et al.* 2011). This may be that these natural response behaviours are the expression of evolved behavioural traits that allow the current generation of selected individuals to modulate behaviours based on the predation threat according to the "risk allocation hypothesis" (Beauchamp and Ruxton 2011). How evolution has shaped these learned behaviours (and correctly identifying appropriate contexts) remains poorly understood, as we are only able to observe individuals with learned risk regimes (in the laboratory controlled experiments), or within natural populations that can also learn predatory patterns within their natural habitat and adjust behaviours within their lifetime. The effect of predators on the plastic response to predators, both directly and through evolutionary time, with little previously learned predatory experience can give insight into the underlying effects predation risk can have on behaviours.

My thesis work looks at the interactions between *Drosophila melanogaster* and two generalist visual predators, the active hunting zebra jumping spider (*Salticus scenicus*, henceforth spider) and the ambush hunting Chinese praying mantid (*Tenodera aridifolia sinensis*, henceforth mantids). *Drosophila melanogaster* has been one of the most extensively used model organisms for behavioural, genetic and developmental research (Dietrich *et al.* 2014, Roberts 2006). Despite this, there is relatively paucity of research with respect to its natural ecology, in particular, the interactions with predators. *Drosophila* are useful as a model for predator-prey experiments, as they are a common prey species to a variety of generalist predators, have a well-defined genome with available genetically manipulated fly lines (Huang

et al. 2014, MacKay *et al.* 2012), and can be reared and maintained within a controlled laboratory setting. With the short generation time for *Drosophila*, there is also opportunity to experimentally evolve flies to experimenter controlled selection regimes, such as predation (DeNieu 2014, Kofler and Schlötterer 2014, Schlötterer *et al.* 2014).

With all these advantages of *Drosophila melanogaster*, I was able to examine the behaviours of wild-caught populations of *Drosophila* with direct predator threat to examine the natural response to predation. As well, using genetically variable lines available (Huang *et al.* 2014, MacKay *et al.* 2012), the variability of antipredator behaviours was also studied. Lastly, the short generations of *Drosophila* allowed the analysis of selection acting on potentially beneficial behaviours due to constant predation exposure over many generations.

How different predation exposure, namely high predation risk and selection by predation, alters the behavioural response of *Drosophila* is examined to determine how well the "risk allocation hypothesis" predicts the behavioural repertoire of flies. I specifically am looking at the effects of predation on three behaviours of *Drosophila*; predator detection and avoidance, overall daily locomotor activity and the mating behaviours of both males and females. This is done with direct predator exposure, while evolved populations with predators as the selective force are used to observe the evolved response for both locomotor activity and mating behaviours. These evolved populations are experimentally evolved *Drosophila* populations undergoing constant (yet relatively low) predation from either spider or mantid predators. These predator treatment populations are compared

with control populations that are without predators but otherwise housed similarly. Predator selection has shown to be causing variation with populations of *Drosophila*, namely for variation in wing shape, emergence times, and survivability (DeNieu 2014, Elliot *et al.* 2017), and will be discussed here for behavioural modifications.

I broadly predict that there will be variation in the response to predators for *Drosophila*, both genetic variation in predator avoidance (1.1.2), and natural variation in activity (1.1.3) and mating (1.1.4) in response to predation. I also expect evolved populations with selection from predators will modulate behaviours to display an overall behavioural pattern that maximizes *Drosophila* fitness, namely activity (1.1.3) and mating (1.1.4). For each specific behaviour (avoidance, activity and mating) I will give a brief overview of the behaviour and the specific expectations for that behaviour, depending on the population.

1.1.2 Predator Detection and Avoidance

Flies that are able to detect a predator and avoid areas with higher risk to prey will be able to survive and are more likely to be selected (Lima and Dill 1990). As noted earlier, early detection is highly beneficial to increase survival of prey. Many advanced sensory structures have been selected within species to increase the detection of predators, whether that is advancement of night vision (Gerkema *et al.* 2013, Walls 1942), behavioural vigilance techniques (Clutton-Brock *et al.* 1999) or the detection of predator chemical signals (Pereira *et al.* 2017, Persons *et al.* 2002)(reviewed by Dicke and Grostal 2001). Primary detection of predators

can lower enable less encounters with predators and reduce any non-consumptive or consumptive effects of predation. Specifically for *Drosophila*, the detection of predators has pointed to both olfactory cues or visual cues playing a major role.

For visual cues, de la Flor *et al.* (2017) studied the response to two species similar to the predators I will be using, jumping spiders, *Plexippus paykulli*, and Texas unicorn mantis, *Phyllovates chlorophaena*. Results indicated that visual stimuli played a major role in predator detection and avoidance, with avoidance unaffected by removing olfactory abilities through mutant strains (*orco* mutants lacking odorant receptors). Once flies were visually impaired, measures of predator detection and avoidance was reduced, thus indicating vision was required primarily for detection of predators compared to olfactory cues. Card and Dickinson (2008) and Wu *et al.* (2016) also found vision played a role in *Drosophila* detecting simulated predator stimuli and the mediating the behavioural response to this threat,

Vision was also found to play a role in detection of predators for *Drosophila* exposed to parasitoid wasps (*Leptopilina heterotoma*, Kacsoh *et al.* 2015a), but this study did not discount any olfactory cues playing a role in detection. For a similar wasp species, *Leptopilina boulardi*, Ebrahim *et al.* (2015) found a specific neural pathway that senses the chemical cues left by wasps to avoid egg laying within these regions. Using a similar mutation as used by de la Flor *et al.* (2017) (*orco* mutants), flies were unable to avoid oviposition on food with chemical cues of predators, or avoid predation cues as larva (Ebrahim *et al.* 2015). The olfactory detection of predators has been selected for within the larva (immediate fitness) and the adults egg laying behaviours (future fitness). Olfaction may be an important process as

well for predator detection that should not be removed from analysis of *Drosophila* interactions with predators, contrary to results from de la Flor *et al.* (2017) with similar predators to the ones that I use.

This response found by de la Flor (2017) may not be a naturally occurring pattern in *Drosophila* but rather due to lab domestication, as the flies used have been without predation for 15+ years. The behaviours displayed by this population may not be reflective of the natural abilities of *Drosophila*, specifically for predator detection and avoidance. Olfactory cues may be important for the detection of predators as well, as the combination of visual and olfactory detection of predators should be the most beneficial evolutionary trait to maximize detection. For *Drosophila*, it is not clear how predators are detected and avoided, and the genetic variability associated with avoidance that would be selected upon in the population.

I predict that there is genetic variation in the ability to avoid predator cues, as detection requires a suite of decisions and sensory structures (olfactory and visual). I use lines of genetically identical flies from the *Drosophila* genetic reference panel (DGRP, 1.2.3) (Huang *et al.* 2014, MacKay *et al.* 2012) and a choice assay. The goal was to test the genetic variability for *Drosophila* avoidance of predator cues. These DGRP lines are fully inbred lineages that display consistent within-line behaviours, and have been found to have variation in olfactory senses (Arya *et al.* 2015). As being in the vicinity of a predator is expected to increase the risk of mortality, I expect an overall avoidance of predators across all lines. I also observe how variable avoidance is, expecting a range for the genetic lines for high avoidance to random assortment, with lines showing consistency between sexes.

1.1.3 Locomotor Activity and Predation Risk

In a high predation risk environment, drawing the attention of a predator can bring upon serious harm to prey and those around them, so modulating these detectable behaviours can presumably reduce predatory mortality (Lima and Dill 1990, Werner and Anholt 1993). Many organisms use strategies to avoid detection by predators (i.e. camouflage, mimicry etc.) (Robinson 1981, Stuart-Fox *et al.* 2008), which also require behavioural modifications to minimize detection and maximize survival. The simplest method to lessen predators detection is reducing the overall activity during predation exposure. This can reduce the chances of detection by predators and increase the chances for survival (Freed 1984, Parigi *et al.* 2014, Prete 1999, Werner and Anholt 1993)

Behavioural decisions to maximize survivability generally depend on the hunting mode of the predators (Parigi *et al.* 2014, Schmitz 2008, Stuart-Fox *et al.* 2008), whether they be actively hunting for prey (i.e. the zebra jumping spiders) or are ambush hunters waiting for prey to come to them (i.e. the Chinese praying mantids). Although these two organisms deploy varying strategies for predator capture, the commonality between the two is the dependence on visual detection of prey when hunting, generally with greater detection of moving prey (de la Flor *et al.* 2017, Freed 1984, Jackson and Pollard 1996, Parigi *et al.* 2014, Prete 1999). Thus, the expectation would be that in the presence of a predator, a reduction in activity would be beneficial with visually oriented predators. The hunting mode as well will play a role in avoiding capture, for example, the ambush hunting mantids depend on prey moving into their vicinity and striking. With a reduced activity, the chances of predation can drop drastically as there would be few opportunities

for capture. Previous studies have observed adjustments in active behaviours of *Drosophila* in response to mantid and spider predatory cues (de la Flor *et al.* 2017, Elliot *et al.* 2017, Parigi *et al.* 2014).

For *Phyllovates chlorophaena* mantids, de la Flor *et al.* (2017) found flies were able to avoid a centralized cage with the mantid predator, however, this experiment did not test if this avoidance was due to avoidance of the center or from motionlessness. For the mantids I use (*Tenodera aridifolia sinensis*), Parigi *et al.* (2014) and Elliot *et al.* (2017) found little influence of mantid cues on fly activity. While looking at the behavioural repertoire of *Drosophila* in the presence of different predators, Parigi *et al.* (2014) found little variation in the behaviours associated with activity (running, walking, jumping etc.) between an individual's times before or after a mantid is introduced. Elliot *et al.* (2017) examined the activity of 5 *Drosophila* (4 males, 1 female) over 96 hours with direct mantid cues (mantid present but cannot feed), but found no substantial variation in activity between treatments without mantids, with a dead or with a live mantid. The active predators were expected to induce a greater active response by *Drosophila* (as seen with spider models, de la Flor (2017)), but no variation was detected between dead (stationary) and alive (presumably active) mantids. Together, these appear to point to our ambush hunting predator not altering active behaviours, both for direct behaviours observed and for the daily activity levels.

In response to a similar species of spiders I use (jumping spider *Plexippus paykulli*), de la Flor *et al.* (2017) found increases in *Drosophila* activity when exposed to a spider, presumed to be a product of the flies searching for escape. The active hunting spiders I use, *Salticus scenicus*, was used to observe how direct

exposure altered a suite of behaviours (walking, running, jumping, etc.) expressed by *Drosophila* (Parigi *et al.* 2014). It was found that flies exhibited more behaviours associated with activity after a spider was introduced to the assay chamber the flies were housed within. This experiment allowed flies to acclimatize to the environment before the introduction of predators, such that any alterations in behaviours should express the plastic response to predation, and not experimenter handling, a possible confounding result for the response found in de la Flor (2017). Together, these experiments point to *Drosophila melanogaster* increase locomotor activity when exposed to active hunting spider predators.

However, the activity of *Drosophila* has been looked at after immediate encounters with a predator and the activity shifts with brief predatory exposure. The initial responses of flies to predators would be indicative of a high stress environment, and these studies did not look at the variation in activity due to predators throughout longer exposure and depending on the light conditions of the encounter. Modulating activity based on the time of day or based on the phase of light or dark could help to increase survivability, and when experiencing predation cues, *Drosophila* may change activity based on the time of day. I looked at data collected by members of the Dukas lab (McMaster University) for *Drosophila* activity over 24 hours, with 12 hours of light and 12 hours of darkness, all while flies experience predatory olfactory cues.

As these predators are visual hunters, the expectation would be some increase in activity (relative to controls) during times of darkness to lessen encounters with predators while attempting to compensate for lost activity during the day. This is because I also expect that during times of light, activity will be reduced

to avoid drawing the attention of the movement sensing predators. Although this response is expected for both predators, due to the evidence of little effect of mantid predators on activity (both with short exposure and longer exposure), additional comparisons with varying controls are not carried out for mantid predators, with primary focus on the effect of spiders on activity.

Using a population of freshly caught flies (see 1.2.2), that should display the natural behavioural response to predation, the measures of daily activity is found for many combinations of cues. As done with de la Flor *et al.* (2017) and pointed out as a possible confounding explanation to their results by Parigi *et al.* (2014), the presence of another large organism may be the cause of activity variability (fear of novel cues, neophobia), and not due to a response to predation risk. We use field crickets and a conspecific (*Drosophila*) as additional controls to the no predation cues, as well as altering the diet of the spiders used in the experimentation, being fed either flies or being fed crickets prior to experimentation. The diet of predators has been shown to be important in predator recognition in prey species, such as damselfly prey (*Enallagma* spp.) decrease behaviours (head bends, feeding) from exposure to pike (*Esox lucius*) with different diets (Chivers *et al.* 1996). I expect that for a the naturally variable population, the response to spiders will be the same, regardless of predator diet.

As I expect flies will reduce activity in order to reduce the detection by predators, a lower activity level should be evolutionary advantageous. Thus, if a population was experiencing predator selection for many generations, the population would be expected to reduce activity overall to a level that is most beneficial for overall fitness. Using populations of flies experiencing predator selection for many

years (see 1.2.4), I examine how predation has altered the activity of *Drosophila* populations. The expected beneficial reduction in activity should be expressed throughout the population, as those that are less active are likely to survive and pass on the beneficial genes for low activity. I expect both spider and mantid selection populations to show a similar trend as the natural population, in that there will be a reduction in activity levels to reduce detection, but a slight increase in night time activity as this would be an opportunity for gaining other fitness benefits (mating or foraging), behaviours thought to increase the risk to *Drosophila*.

1.1.4 Mating Behaviours Under Predation Risk

When predation occurs, different fitness components may be constrained by one another, such that there is a trade-off between avoiding predation (vigilance) and resource acquisition, which includes reproductive output (Candolin 1998, Franklin *et al.* 2014, Koga *et al.* 1998, Magnhagen 1991). Many sexually selected characteristics have evolved as a method to increase reproduction, but these can be at a cost to survival as these traits can draw predator attention. Therefore, it is behavioural modifications that can be utilized as a method to lessen predation by either spending more time vigilant or by reducing the chances of detection (reduced detectable behaviours etc.) (Lima and Dill 1990, Magnhagen 1991).

Some individuals will avoid courtship and mating opportunities when encountering predators to focus on immediate survival at a potential cost to future fitness

(mating opportunities), such as male three-spined sticklebacks (*Gasterosteus aculeatus*, Candolin 1998) or the fiddler crab (*Uca perplexa*, Koga *et al.* 1998). Other species alter investment in particular behaviours that while not putting off mating, the overall time spent and energy invested into mating behaviours is reduced. For example, some species (such as the wolf spider *Hygrolycosa rutbrofascia*) reduce courtship calls to lessen predator detection (Katiaho *et al.* 1998), while others reduce choosiness in order to gain benefits from mating while reducing the risk of searching for mates and engaging in risky behaviours, such as the amphipod crustacean (*Gammarus duebeni*, Dunn *et al.* 2008) or tungara frogs (*Physalaemus pustulosus*, Bonachea *et al.* 2011). Apart from increasing the time that can be spent engaging in antipredator behaviours, these adjustments can reduce overall movement, noise or grouping of prey, which can decrease the probability of detection by predators (Kemp 2012, Katiaho *et al.* 1998, Lima and Dill 1990, Magnhagen 1991).

Very little work has observed *Drosophila melanogaster* mating behaviours when exposed to predation threat. Work with *Drosophila* and a parasitoid wasp has shown females reduce post mating behaviours in response to predation, showing reduced egg laying in areas exposed to a wasps (larval predators) (Kacsoh *et al.* 2015a, Kacsoh *et al.* 2015b). These adult female flies are not in direct threat of predation and the risk to immediate fitness has no impact on overall fitness. But as these parasitoid wasps predate upon larva, there can be lost offspring which would reduce any potential future fitness benefits. If the adults were to lay a large quantity of eggs, these offspring are less likely to survive to sexual maturity, meaning it is more beneficial for the adults to find food patches with

lower predation threat to offspring.

While studies with larval predators (parasitoid wasps) can show how *Drosophila* alter post mating behaviours in response to threats to future fitness, the response to immediate fitness threats (i.e. survival) is a major goal of my study. Specifically I am interested how courtship and copulation is altered with predator exposure, as I believe these behaviours put *Drosophila* at an increased risk for predator detection/capture. Experiments with *Drosophila* have shown flies can modulate mating behaviours based on the environmental conditions they are presented with. This is seen with male *Drosophila* reducing courtship latency based on previous courtship experiences (Dukas 2005), males reducing courtship intensity and latency based on exposure to mated females (Noor 1997) or males increasing copulation duration due to interactions with rival males (rivals for access to females) (Bretman *et al.* 2009, Nandy and Prasad 2011, Nandy *et al.* 2016). This behavioural shifts in courtship and copulation allows for an increase in the reproductive output flies, and highlights that the environment conditions can elicit a plastic response in mating behaviours. My main interest is if a similar pattern is expressed with risk to the flies immediate fitness due to predation risk.

When encountering a female, a male *Drosophila* engages in a stereotypical courtship display and attempts copulations. These displays involve many distinguishing behaviours, where a male will orient to females and follow the female while engaging in a courtship wing song, until the female is receptive. The male then engages in genital licking and attempts copulations with the female (Hall 1994, Lasbleiz *et al.* 2006, Spieth 1974).These displays are expected to increase the risk of a mating pair in the presence of a predator (Candolin 1998, Lima and

Dill 1990). Apart from taking time away from vigilance, which will not allow for avoidance of predators, these behaviours are expected to draw the attention of the predators as there is both auditory and active behaviours. It may be that the auditory wing song can draw the attention of predators (as in *Hygrolycosa rutbrofascia*, Katiaho *et al.* 1998), or the array of movements may draw the attention of visual hunters (Freed 1984, Jackson and Pollard (1996), Parigi *et al.* 2014, Prete 1999). Copulation would also be risky, as the combination of two *Drosophila* would be more detectable by predators, and the duo would be much slower if escape is necessary, similar to plague locusts (*Chortoicetes terminifera*) having increased mortality as a mating pair (Kemp 2012).

Using the zebra jumping spider predator (*Salticus scenicus*), I expect that a male will reduce the overall time spent courting a female, and reduce courtship attempts displayed to a female when a predator is present. This was looked at with a recently caught wild population of *Drosophila melanogaster* (1.2.2) with the belief that courtship would increase predator detection, and males will focus primarily on immediate fitness. A male and female that engage in copulation are also expected to be at a greater risk to predators, as the pair will move slower and be a larger target for detection. With this expectation of increased risk, a reduction in copulation occurrence is also expected. Any copulations that do occur are expected to be brief in duration and females would display less hesitation and mate quickly to lessen the time spent with a male possibly drawing attention to the pair.

In an evolutionary context, the ability to avoid predation while gaining mating will be beneficial to individuals under long term predation exposure. As I predict

reduced courtship displays and less time spent copulating will increase adult flies survival when under predation risk, I also expect evolved populations with predation risk (1.2.4) to show a similar response. Being under constant risk of predators, populations of evolved flies with predation should express traits that reflect the greatest fitness benefits. First, I expect that flies are able to mate at a younger age. As the predators used predate upon adult flies regardless of sexual maturity, individuals that mate earlier in life may be able to achieve reproductive output before the possibility of death. I looked at data collected by Abhijna Parigi (Michigan State University, MSU), measuring the mating times and copulation counts for populations of selected flies. These flies are from different age categories, used to determine the peak age for mating behaviours to occur between treatments. Measures of courtship latency, copulation latency, duration and occurrence was made for each age category. The expectation was that in order to spend more time vigilant to threat, populations selected upon by predators will show a reduced latency for courting and copulating, copulate for a shorter time, and copulate in a greater frequency at younger ages compared to control populations.

Night may be a safer time to express risky behaviours, as expected with activity (1.1.3) and mating behaviours. The visual hunting predators used within the evolved population cages (1.2.4) should have a more difficult time detecting prey (i.e. movements of a courting male) while it is dark compared to the day. The Dukas lab (McMaster University) measured the courtship time of populations under either red light (night) and normal ceiling lights (day) to compare the relative amount of time exhibiting courtship behaviours, expected to increase in mantid and spider selected populations during the dark times compared to the control

populations. Mating is expected to increase the risk to *Drosophila*, and modulating behaviours under direct threat and after being evolved with predation risk will increase both the immediate and future fitness effects of flies.

1.2 General Methods

Three different populations of *Drosophila melanogaster* flies were used throughout experimentation; a population of recently caught wild flies with little adaptation to the laboratory environment (Baxter *et al.* 2015), 60 lines from the *Drosophila* genetics reference panel (MacKay *et al.* 2012), and 12 experimentally evolved populations of flies undergoing different predator selection regimes (DeNieu 2014, Hangartner *et al.* 2017).

1.2.1 Predators Used For Experimentation

Two generalist predators of *Drosophila melanogaster* are used throughout experimentation, the zebra jumping spider, *Salticus scenicus*, and the Chinese praying mantids, *Tenodera aridifolia sinensis*. The zebra jumping spiders were collected along warm sunlight walls around Michigan State University and McMaster University campuses, and regions of Southern Ontario. The spiders were housed individually in vials at 21°C, ~60% humidity and a 12:12 light:dark cycle. Spiders were fed laboratory populations of flies, unless in use within population cages or prior to any experiments requiring a specific feeding regime. The Chinese praying mantids were brought into the laboratory as egg cases, either collected

from fields in Southern Michigan or purchased from Nature's Control Oregon (http://www.naturescontrol.com/). The egg cases were stored at 4°C, and removed as needed to replenish the stock of mantids. Individual egg cases were removed and left at room temperature until eclosion. These mantids were housed in a 32.5 cm^3 mesh cages (Bugdorm-43030) and fed and maintained similarly to the spiders.

1.2.2 Population of Recently Captured Flies

This population of flies was collected in 2014 in Southern Ontario and maintained in large population cage at 25°C, 60% humidity and 12:12 light:dark cycle (lights on at 10:00 am) on a sucrose-cornmeal medium with live yeast (see General Methods: Baxter *et al.* 2015). Experimental flies were reared at similar conditions, with one exception. For the courtship and copulation analysis with direct predator cues, this population was housed with lights on at 8:00 am, with 12 hours of light, and reared on a molasses-cornmeal medium (with live yeast) prior to experiments.

1.2.3 *Drosophila* Genetic Reference Panel (DGRP) Lines

The *Drosophila* genetic reference panel (DGRP for short) is a collection of over 200 inbred (20 generations of full-sibling mating) *Drosophila melanogaster* fly strains initiated from isofemale lines collected in a Raleigh, North Carolina Farmer's market (See Huang *et al.* 2014 and MacKay *et al.* 2012 for population details). In a study by Zwarts *et al.* (2015), severe neural defects were found

within pure DGRP lineages, specifically in the mushroom bodies. These neuron defects caused by inbreeding depression are expected to have profound effects on behaviours compared to wild caught flies. This may effect neuronal activity important to avoiding predators, such as the olfactory sensory neuron (OSN), used to detect chemical signals from parasitoid wasps (Ebrahim *et al.* 2015). To combat inbreeding depression, DGRP dihybrid lines were created by crossing 59 different DGRP lines with one common "tester" line, DGRP line 83, selected for its high fecundity. For each of the 59 lines, five males were placed in a vial with five virgin females from line 83 and the F1 offspring of this cross (dihybrids) were used for experimentation. All lines and dihybrids were maintained on a 12:12 light:dark cycle (lights on at 8:00am) at ~60% humidity and 25°C within vials on a molasses-cornmeal medium (+ live yeast), with line 83 housed in bottles as well due to the increased numbers of females required for crosses.

1.2.4 Experimentally Evolved Populations with Predation Selection

In 2010, wild *Drosophila melanogaster* were collected at Fenn Valley vineyard in Southwest Michigan as a starting population for experimental evolution. After splitting this population into twelve 32.5 cm^3 Bugdorm mesh cages (~1500 flies per cage), each was designated a treatment. Four populations were controls with no predators present, four populations were continuously exposed to ~30 zebra jumping spiders and four populations were continuously exposed to ~30 1st instar Chinese praying mantids, with new predators cycled throughout. Each population

of overlapping generations was maintained on a 12:12 light:dark cycle at ~40% humidity and 24°C initially (for age-dependent mating experiments at Michigan State University; 1.3.3), and moved buildings/growth chambers in 2015 to McMaster University, housed at ~60% relative humidity and 21°C (for activity analysis (1.3.2) and light:dark mating assays (1.3.3)). Each population was given 200 mL molasses-cornmeal-yeast food bottles, which was added every 5 days (removing the oldest bottle after 25 days), and moved to clean cages monthly. As these populations have overlapping generations, there is no exact measure of the generations of evolution to have occurred, but is estimated to be ~16 generations per year.

1.2.5 Statistical Analysis

While there were distinct models fit for each analysis depending on the behaviours measured and the populations used, there are several important processes and packages used throughout. Each experimental design is laid out in separate sections (1.3) with the analysis for each within these sections. Analysis was done in R version 3.3.2 (R Core Team, 2016). For fitting (generalized) linear mixed models, the lmer() and glmer() functions were used ("*lme4*" package version 1.1.12, Bates *et al.* 2015). The "*tidyverse*" set of packages (version 1.1.0, Wickham 2017) was used for data cleaning and plotting, and the Anova function ("*car*" package, version 2.1.4, Fox and Weisberg 2011) was used for p-values and Wald Type II Chi-Square values. Each experiment required specific definition of fixed and random effects, and although some experiments that were completed on separate days included measures of humidity, temperature and barometric pressure, these were not ultimately included in the models, as the random effect of day encompassed

these effects. Indeed including the random effect of day along with the fixed effects of humidity, temperature and pressure resulted in something analogous to multi-collinearity and resulted in poor convergence of models. While not shown, the results for the biological effects of interest were largely similar whether random effects of day were included or alternatively the fixed effects of day to day variation in temperature, humidity and barometric pressure.

1.3 Experimental Methods

1.3.1 Measuring Variability in Predator Detection/Avoidance by *Drosophila*

To measure the degree to which *Drosophila* could detect and would choose to avoid spiders, flies were put into a 355 ml *Snaptite* container and given a choice between two 4.5 cm high vials, one with a spider predator present and one with no predator within. Each vial had a small layer of regular *Drosophila* media (0.5 cm) and a cut syringe tip on a lid to act as a funnel which allows flies to enter a vial, but not exit. Using the 59 DGRP dihybrid lines created in a cross with line 83 (1.2.3), adult offspring were collected and housed for two days at a 10 male to 10 female sex ratio, allowing for social and sexual interactions. Males and females were then separated into separate sex vials for 2 days. Approximately two hours before the addition of flies to the containers, the two vials were placed into the *Snaptite* container with one vial housing a spider predator, noting the location of

the spider vial (front vs. back, Fig. A1.1). Ten same sex individuals were added randomly to a container and left for two days to enter either vial.

After 48 hours, the location of each fly was recorded (vial with spider, vial without spider, outside vials), and only those containers with at least six flies inside vials were retained in the analysis. As the number of trial containers was limited (112–120 containers per day depending on spider availability, Fig. A1.2), the experiment was completed over 10 blocks, with each DGRP lineage done each day (1 male and 1 female replicate per line per day) to avoid confounding line effects with day effects. The temperature and humidity, along with the change in barometric pressures through the 48 hour period was monitored, however the random day effects was highly correlated with these measures, and these effects not included in analysis, and accounted for by the day effects.

Three generalized linear mixed models (package "*lme4*") were used, in particular a logistic model and a comparison of complex to simpler mixed models using a parametric bootstrap method (R package "*pbkrtest*", V.0.4.7). The full logistic mixed model

```
glmer(cbind(Spider, Not_spider) ~ 1 + Sex +
    Spider_Location + Row + (1 | Date) + (0 + Sex | DGRP))
```

was fit for a response of flies found with a spider ("Spider") or not with a spider ("Not_spider"), having sex as a fixed effect, and using date as a blocking random effect. The location of the spider vial (front vs. back; "Spider_Location", Fig. A1.1) and the row of the container on the table (Fig. A1.2) were included as well.

This model also allows the effects of each DGRP line mean and sex effects to vary randomly ("0 + Sex‖DGRP").

A Wald Type II Chi-square was used to evaluate the fixed effect of the intercept (to determine if there was an overall bias in the location a fly was found, as well as the effects/ correlations of sex. To asses the random effects, and in particular whether evidence is present for genetic variation in DGRP lineages, a parametric bootstrap was done. Specifically, a comparison between two models was completed, looking at the full model above compared to the removal of sex random effects, or the removal of both sex and DGRP random effects. These results are displayed with the PBtest p-value for the associated model comparisons, comparing the test statistic with the observed result.

1.3.2 Measures of *Drosophila* Activity with Different Olfactory Stimuli

All activity data collection was done using a ***D****rosophila* **A**ctivity **M**onitor (DAM; Trikinetics, Fig. A1.3) by members of the Dukas laboratory at McMaster University, specifically Dr. Dukas and Erik Etzler. Two monitors were used (fixed blocking effect within model), with 32 wells each to hold a total of 64 vials for one 24 hour measure of activity. The **DAM** counts the total times a "focal" fly crosses a laser that sits at the middle of a vial each minute over the experimental duration. These monitors were held within an undisturbed humidified chamber at 25°C with controlled hours of light (10AM) to dark (10PM). These monitors can hold small vials (22mm wide by 48 mm long) that have a snap cap lid (with

ventilation hole). After the addition of ~4 ml of food (12 mm high), there is a 7mm gap between the food and the laser that will record activity.

Many different combinations of only olfactory cues was used for these experiments, using a combination of zebra jumping spiders, Chinese praying mantids, field crickets and con-specific "stimuli" flies. Mantid cues were compared with a control of no cues present, comparing the effect mantid olfactory cues have on "focal" fly activity compared to no predator present, similar to Elliot *et al.* (2017) and Parigi *et al.* (2014). Spiders olfactory cues effect on *Drosophila* activity was compared to many variable cues, including spider olfactory cues compared to no cue controls, like with mantids. To ensure any effect seen when comparing spider cues vs. no cues is not a product of any novel olfactory cues present in the vial (neophobia), the flies activity was recorded when exposed to spider or cricket olfactory cues. Lastly, a 4 way comparison between cricket cues, "stimuli" fly cues, and 2 cues from spider with different diets (cricket or fly diet) was also used. Here the effect on activity by con-specific olfactory cues (i.e. "stimuli" flies) can be observed, as well as the effect spider diet (spider fed flies or spider fed crickets) has on the fly activity, while controlling for potential neophobia (crickets). Each comparison above was completed together over 24 hours in two monitors, generally ranging between 12–16 flies assayed for each treatment.

All stimuli (cues) were acclimated for 3 days prior to experiment, with a spider, mantid or cricket housed singly within vials, and "stimuli" flies acclimated as a trio (all males). On the third day of acclimation, the "cue" individuals were removed along with any accompanying silks from spiders, leaving only the olfactory cues within the vials. One live sexed male "focal" fly from the wild caught population

(1.2.2) was aspirated into the vial. Recordings count activity over 24 hours and the data is outputted as a minute by minute count of activity for each vial.

When analyzing the activity of the evolved populations (1.2.4), the trait of interest was not the plastic response to olfactory cues, but rather the evolved activity between populations. Measures of activity was completed similarly to those above, in vials with small amount of food medium, but all populations and vials were experiencing no cues. After 2 generations reared in bottles without predators present (to remove any plastic or maternal effects due to predation), each population was recorded in the **DAM**. As there were many more replicates and populations of flies to measure, this was repeated in many blocks of 3 replicates for two treatments and 2 for the other treatment (due to 32 wells of the **DAM**), alternating the 2 replicate population per block, which resulted in 240 individuals assayed (80 per treatment).

Each comparison of activity between cues/ populations was run through linear mixed models ("*lme4*" package), accounting for treatment and light as fixed effects while accounting for monitor and individual fly effects. The evolved populations activity models also accounted for the four replicates within each predation treatments (population), as well as the start day (as several blocks were run). Each individual effects on activity was treated as a random effect (including the variation individuals had in response to light) to account for the longitudinal nature of the data.

```
1 glmer(Hourly_activity ~ sin(hour2) + cos(hour2) + Treatment
      + Treatment:Population + light + light:Treatment +
    start_day + monitor + (1 + light | individual))
```

To account for the effects of circadian rhythm on activity, two methods were used, either imposing a circadian periodicity (sin(pi * hour/12) + cos(pi * hour/12)) or fitting a natural smooth cubic spline with 5 knots (ns(hour, 5)) for hours. As both methods showed similar results with respect to the estimated parameters of interest (results not shown), I present the results of the simpler constrained periodicity for hourly effects (model shown above). For locomotor activity results, the parameters of interest were the fixed effects of treatment, and as such, the Type II Wald Chi-Square value and accompanying p-value are displayed, as well as each treatments estimated variation with standard error for these model terms.

1.3.3 Observing the Influence of Predators on Mating in *Drosophila*

For the four experiments below, two were completed by myself (P.K.) using the recently caught population (1.2.2) , while the other two were completed by Abhijna Parigi (A.P.), working with the evolved population behaviours (1.2.4) with different aged flies at Michigan State University, and Dr. Reuven Dukas (R.D.), working with the evolved populations (1.2.4) after the move to McMaster University, measuring courtship times in the light and the dark.

Courtship Behaviours

Using immature females (under 24 hours old), male courtship displays were recorded. Immature females will reject all male copulation attempts (Manning

1967) meaning the females should behave similarly regardless of predation. This allows for recordings of only males adjustments of courtship strategies in response to predation without any confounding effects of female acceptance or rejection behaviours. Using the recently caught population of flies (1.2.2), reared on molasses food medium, virgin male flies were collected via aspiration. These males were housed individually for two days, allowing for no social or sexual interactions prior to the experiment. After clearing all flies in the morning before the planned experimental day, virgin females were collected in the afternoon and housed with 5 females in a vial with live yeast. Both males and females were housed at 25°C, 60% humidity and 12:12 light dark cycle prior to the experiment. At the flies regular "sunrise" (8:00 am) a male was added (via aspiration) to a modified 4 cm wide petri dish that has a layer of mesh separating the flies (on top) and a spider predator (if present) on the bottom. The spider petri dishes had the spiders acclimated 12 hours prior to the addition of the mating pair. The female was then added to the petri dish (via aspiration) and placed in an enclosed bin with a video camera recording from above (Fig. A1.4). Video recordings were completed for 64 mating pairs for each treatment using Logiteck C920 HD Pro webcams, recording four chambers for 15 minutes for each assay (Fig. A1.5). Recordings were scored using a custom script which allows for convenient input of courtship initiation and duration. The same model layout was used for both courtship proportion and courtship counts (below, changing the response variable), which had the primary interest on the fixed effect of treatment (predator vs. no predator), with date used as a random effect that encompasses daily humidity and temperature (as in 1.3.1).

```
1 lmer(court_prop ~ Treatment + (1 | Date))
```

Results display the estimated change in the proportion of time courting, and the number of courtship bouts in 15 minutes (± standard error) as well as the Wald Type II Chi-Square and p-values.

Copulation Behaviours

Following a similar protocol to recording courtship behaviours, copulation times were recorded for the wild caught population (1.2.2). Here 2 same aged virgin flies (2 days old) were used, either housed individually (males) or with 4 other flies (females). As the peak receptivity for flies is ~3 days old, 2 day old males and females were used expecting moderate receptivity to copulation for the mating pair (Manning 1967). Video recordings of mating pairs was done for 30 minutes (or until the end of copulation) above four 4 cm petri dishes within an enclosed bin with either spiders in the modified petri dish or no predators (Fig. A1.4 and Fig. A1.5). These videos were manually scored for copulation latency and duration, and analyzed identically to the model above, with the exception of glmer() needed for copulation occurrence due to the binomial data. Results are also displayed similarly to courtship analysis. Eighty individual mating pairs were used for analysis of latency and duration, accounting for a little over 50% of the individual pairs assayed for copulation occurrence (70 recordings for each treatment).

Courtship and Copulation Measures in experimentally evolved lineages

Using the predatory evolved populations (1.2.4), the evolved mating times after several years of experimental evolution with (and without) predators was examined, looking for evolved shifts in peak receptive age as well as shifts in overall behaviours (A.P.). To remove any maternal effects and direct predator cues experienced by larva, populations were allowed to lay eggs in a bottle, which was then removed from the predation environment into a separate incubator with no predators present. 100 adults from the F1 generation were collected and placed in a fresh bottle, allowing egg laying for 24 hours. This process was continued to have fresh F2 individuals eclosing every 2 days for 14 days of collections. These F2 offspring were used in experiments, having adults with no predatory exposure.

The virgin males and virgin females collected were placed into 4 age categories (age bins by days old: 1) 1-3, 2) 4-7, 3) 8-11 and 4) 12-15). After allowing the female to acclimatize for 5 minutes within the mating vial, a male (from the same age bin) was added and the start time was recorded. The first instance of courtship was recorded and copulation start and end times were also recorded, all through visual scanning of vials. After ~3 hours, if no copulation occurred, the female was labeled as non-receptive. Ten pairs for each treatment-population-age bin was completed, for approximately 360 total pairs analyzed.

Here I modeled the fixed effect of both treatment and age category of flies (and there interaction) with linear mixed models, with replicates of treatments as a random effect along with date. This was done for courtship latency, copulation latency, copulation duration (example below), and copulation occurrence.

```
1 lmer(Rel_Cop_dur ~ 1 + Treatment * AgeBin + (1 | Date) + (1
      | Treatment:Rep))
```

The analysis of the copulation counts (using glmer() as above) displayed an error due to the 100% success of one treatments/age to achieve copulations. This was believed to be due to a perfect separation issue, a computational error where one predictor variable perfectly predicts a response variable. The results for glmer() were similar to a much more simple glm() removing random effects in order to ensure this error is not altering my conclusions, and the results for the glmer will be displayed. Results for all models are displayed as the estimated change (± standard error) for each treatment and age bin response, as well as the population effect, displaying Wald type II chi-square and p-value.

Courtship Behaviours of experimentally evolved populations under Light and Dark regimes

For a more comprehensive analysis of the evolved populations (1.2.4) courtship behaviour, an additional experiment focusing primarily on courtship was completed (R.D). This experiment was done to determine the proportion of time each population spent courting during the day (photophase) and night (scotophase). One male (aged ~24 hours) was placed in a vial with two immature females (less than 20 hour old that will reject copulation) (Manning 1967). Under red light, the courtship times were recorded for night time mating displays, and under normal ceiling laboratory lights, the daytime courtship times was recorded. Recording

(with Logitech webcameras) in 15 minutes sessions for each replicate, the proportion of time spent courting for each male was calculated using a custom recording script for 187 mating pairs (~60 per treatment) over 5 days of experiments. Of interest was the proportion of time courting due to effects of treatments (evolved with mantids, spider and with no predators as controls) and the light phase (light or dark), as well as the interaction between the two, while including the observer effect.

```
1 lmer(P_court ~ Treatment*Phase + Observer + (1|Day) + (1|
    Treatment:Population))
```

The day effects from completely balanced blocks, along with the population replicates were treated as random effects. Results display estimated changes between treatments for light or dark (± std. error) and the Wald Type II Chi-square/ p-value.

1.4 Results

1.4.1 Despite Genetic Variation in Predator Avoidance, no Evidence for an Overall Avoidance Response

There was no overall population level trend among DGRP dihybrid lineages for predator avoidance (N=961, chisq=1.04, p=0.31). Measures of the location of the spider within the container showed an estimated difference between front/back vial of 0.29 (±0.045, chisq=42.27, p<0.00001). Row showed a significant effect (chisq

= 18.27, p<0.0004), with row 4 (closest to wall, +0.26, ±0.067, p<0.0001) and row 2 (+0.15, ±0.06, p<0.011) showing significant differences from row 1 (front row), and row 3 showing no difference (+0.048, ±0.065, p=0.46).

When using the parametric bootstrap between models taking into account DGRP lines as random effects and one without DGRP effects, there is evidence genetic variability is present between lines around the population mean (nsim=500, PBtest p<0.003). When comparing female variation (Fig. 1.1) and male variation (Fig. 1.2) among lineages, we see a negative correlation between males and females (-0.42, r^2=0.31, Fig. 1.3), with an estimated difference of ~0.05 (±0.08 standard error). When we compare sex and DGRP random effects against line (DGRP) variation, we also find significant variation among lines and sexes (nsim=500, PBtest p<0.002).

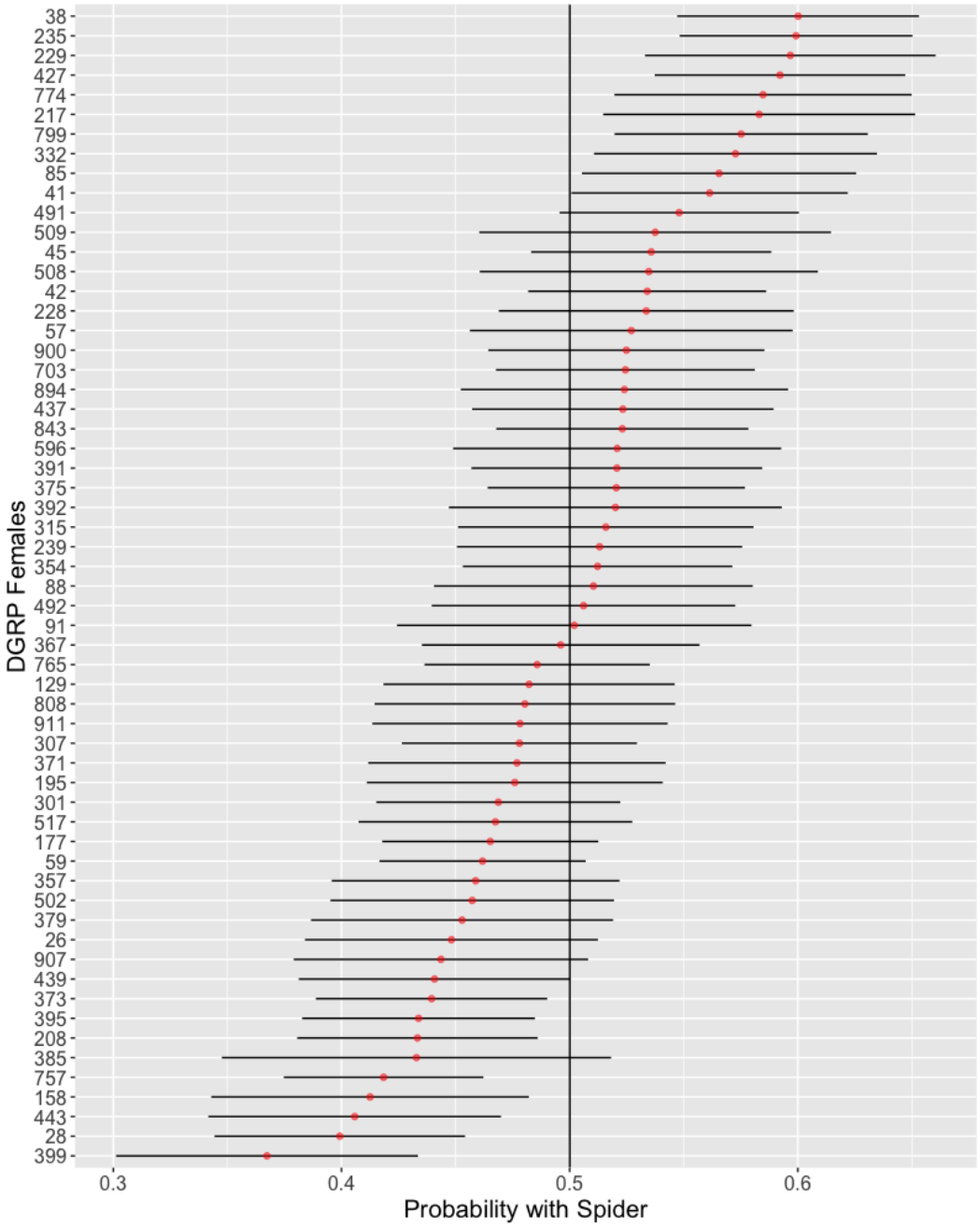


FIGURE 1.1: Variation in 59 **female** *Drosophila* genetic reference panel (DGRP) dihybrid lines. Variation is the probability of being found within the spider vial for each line, with the subsequent increase/decrease around the black line (population mean) due to variation of line effects. Error bars represent 95% confidence interval.

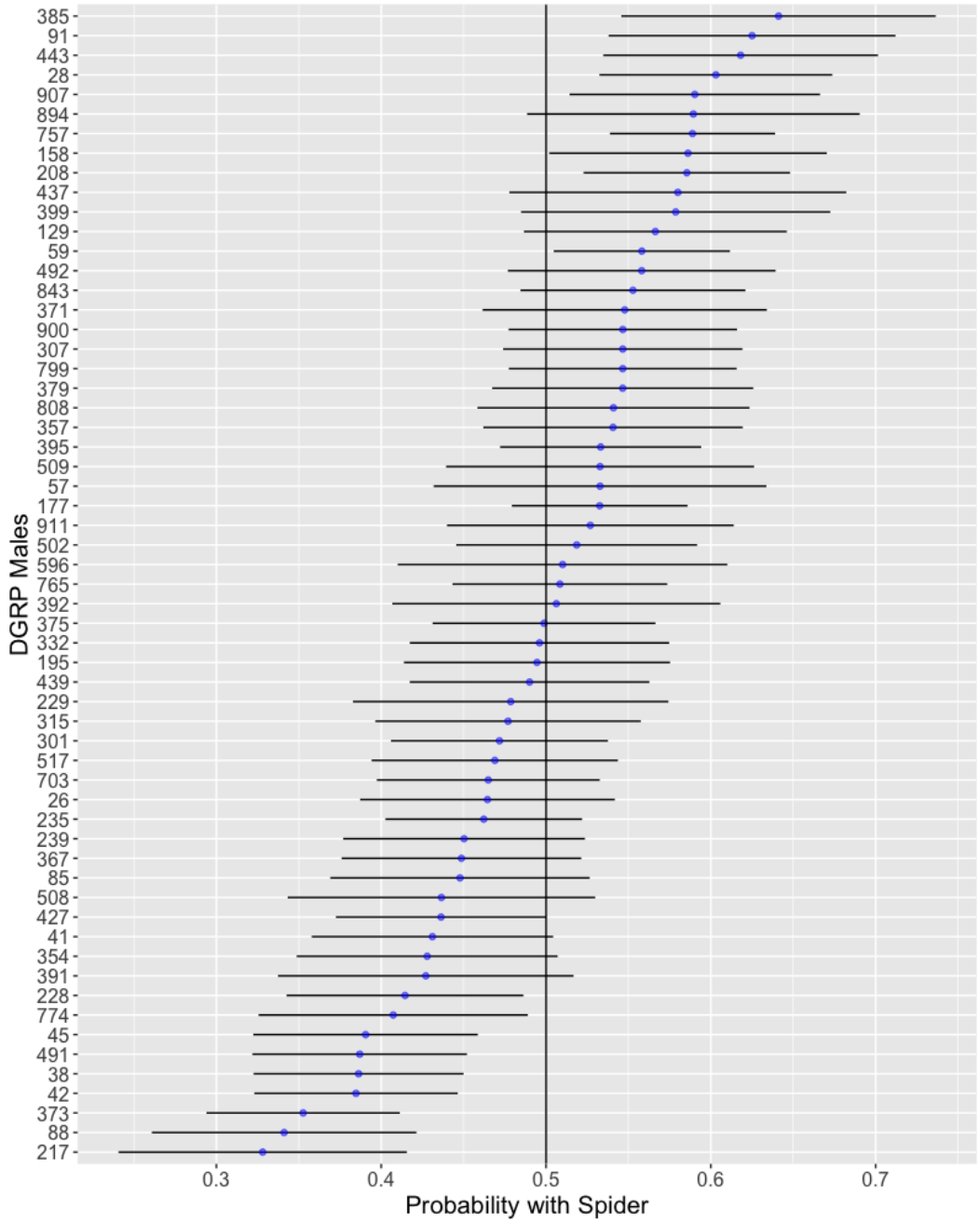


FIGURE 1.2: Variation in 59 **male** DGRP dihyrid lines for the probability of being found with a spider, with line effects causing deviation from the population mean (black line). Error bars represent the 95% confidence interval.

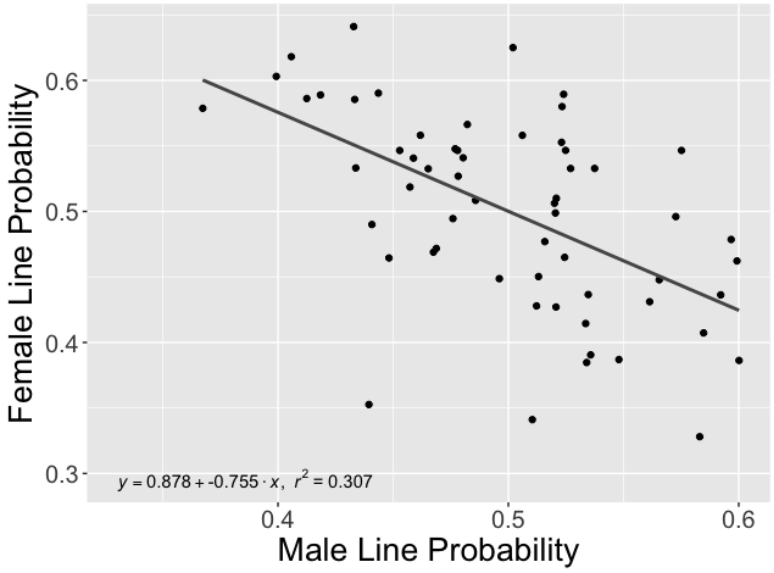


FIGURE 1.3: Correlation between best linear unbiased predictors of different lines for males and females with a negative correlation found between males and females (-0.42). Plot includes the equation fit for correlation line (lm) and the associated r^2 value.

1.4.2 *Drosophila* Express Complex Alterations of Locomotor Behaviour to Predation Cues

Activity of recently established Population

The comparison between *Drosophila* activity when exposed to mantid cues and no predator cues for the recently caught population showed a non-significant increase in activity counts with mantid cue exposure (+2.99 ± 4.5, chisq = 0.081, p=0.78, Fig. 1.4). As expected, the light phase (scoto vs. photo phase) showed a significant increase in activity for both control and mantid treatments in the light compared to the dark, but the interaction between treatment and light phase was not significant (chisq = 2.33, p=0.13), with an estimated decrease of -9.437 ± 6.18

with mantid olfactory cues (Fig. 1.5).

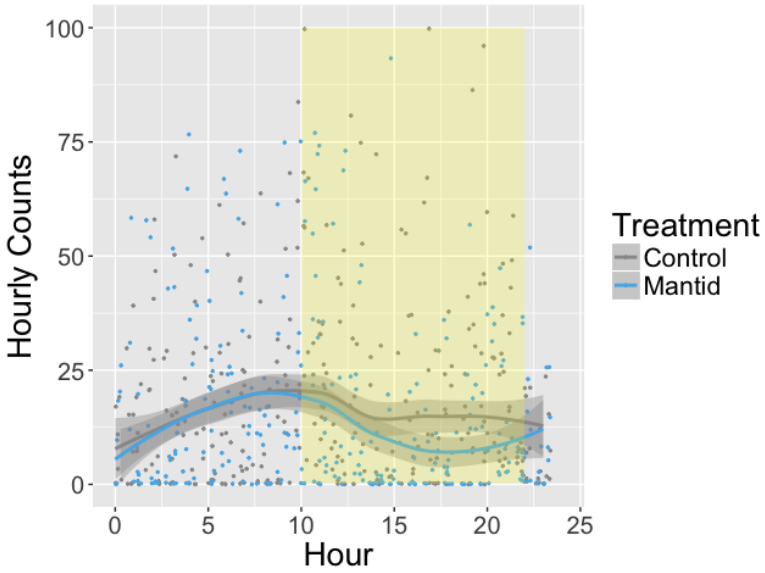


FIGURE 1.4: Hourly activity of male *Drosophila* over 24 hours between vials conditioned with mantid cues and no cue control vials. The yellow rectangle represents the lights being on (10AM to 10PM) and the shaded region around the line represents the 95% confidence interval for this loess spline.

In the presence of cues from the zebra jumping spider, there appears to be a significant reduction in *Drosophila* hourly activity compared to no cues present (chisq = 5.14, p<0.025, Fig. 1.6). Spider olfactory treatments caused an estimated decrease in hourly activity counts of 19 ± 10, but no significant interaction between the phase and the treatment was found (-4.12 ± 9.66, chisq = 0.182, p = 0.67, Fig. 1.7).

To assess whether this effect is due to neophobia (response to any novel cues present, not just due to predator cues), a comparison between spider cues and a control cue from crickets was done. Spider cues showed an estimated increase in *Drosophila* activity (i.e. crossing of **DAM** laser) of +15.62 ± 12.1 for hourly

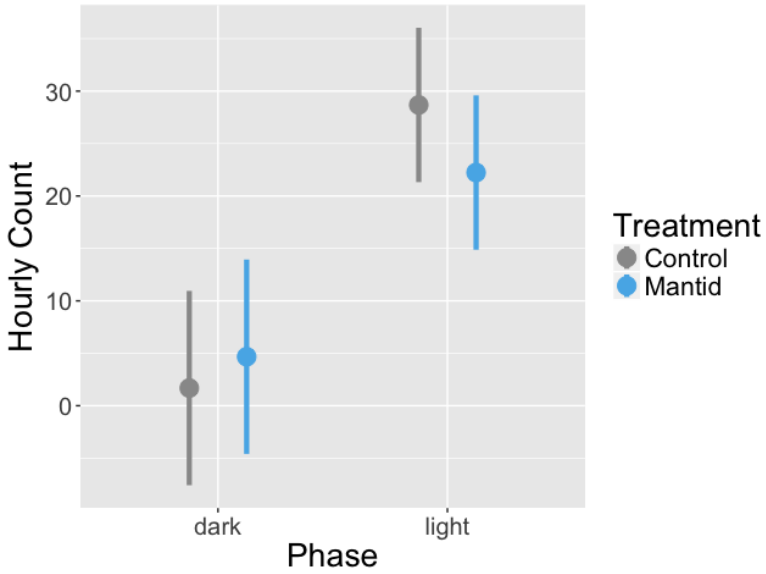


FIGURE 1.5: Hourly activity counts of *Drosophila* made during the light or dark phase of the 24 hour experiment when exposed to mantid cues compared to no cue controls, with error bars representing the upper and lower 95% confidence interval.

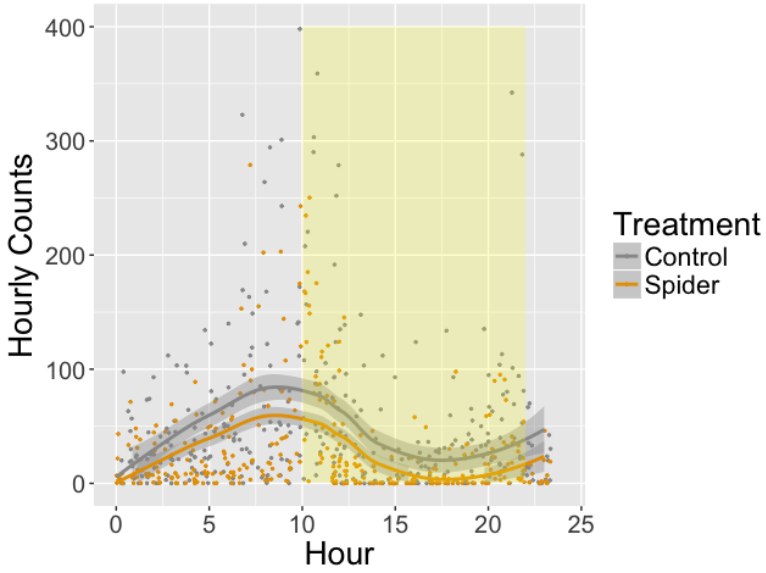


FIGURE 1.6: Hourly activity counts for male *Drosophila* over 24 hours between vials conditioned with spider cues and vials conditioned with no cues. Lights were on from 10AM to 10PM (yellow rectangle) and the shaded region around the line represents the 95% confidence interval for the line of best fit (loess spline).

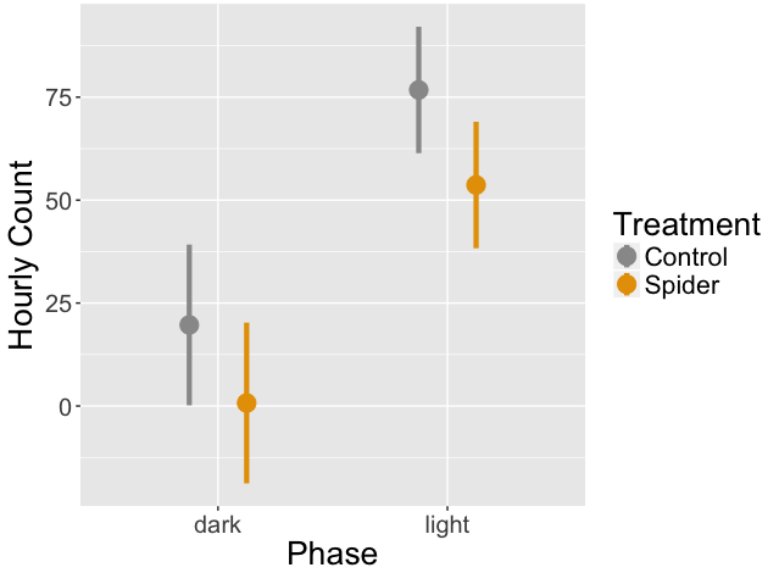


FIGURE 1.7: Light and dark hourly activity counts over a 24 hour experiment for spider cues compared to no cue controls, with error bars representing the upper and lower 95% confidence interval.

activity compared to cricket cues, however this change was not significant (chisq = 2.97, p = 0.085, Fig. 1.8). The treatment by light interaction had less of a change for spider cues (+5.22 ± 8.58) and was also non-significant (chisq = 0.37, p = 0.54, Fig. 1.9).

An additional comparison was done comparing cues from crickets, cues from male con-specific *Drosophila*, and cues from either spiders that were fed crickets or spiders that were fed flies. Overall, there was significant variation found on the treatment level (chisq=8.26, p<0.035, Fig. 1.10) and the treatment by light interaction (chisq= 12.12, p<0.0075, Fig. 1.11). Compared to the crickets estimated mean, there was a decrease for the other three treatments, with both spider treatments acting approximately identically (spider fed cricket (SC); -32.45 ± 20.58, spider fed flies (SF); -32.12 ± 20.86). The fly olfactory cues (F) showed slightly

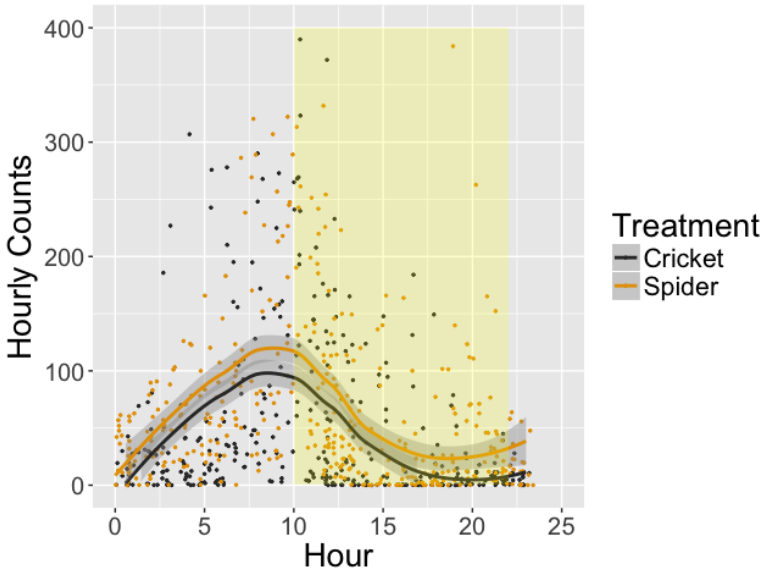


FIGURE 1.8: Comparison between spider and cricket olfactory cues on the hourly activity of *Drosophila* over 24 hours, with times of light (10AM to 10PM, yellow region) and times of dark (10PM to 10AM). Lines represent the treatments loess spline, the line of best fit for the activity trends throughout the 24 hours, with shaded regions representing the 95% confidence interval.

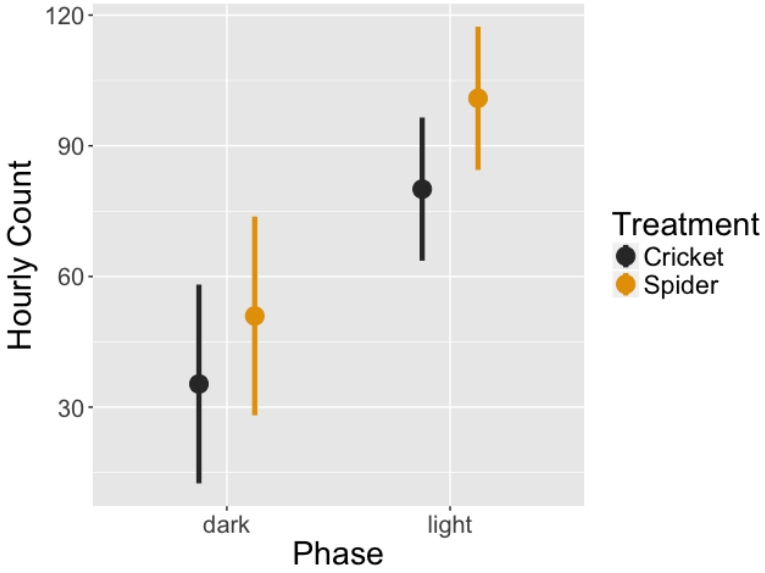


FIGURE 1.9: Hourly counts for light and dark activity over a 24 hours for spider cues compared to cricket controls, with error bars representing the upper and lower 95% confidence interval.

more of a decrease (-45.84 ± 20.86). For the change with times of light, all three showed an increase compared to the cricket mean (F: +26.94 ±14.2, SC: +33.82 ± 14.01, SF: +48.70 ± 14.2).

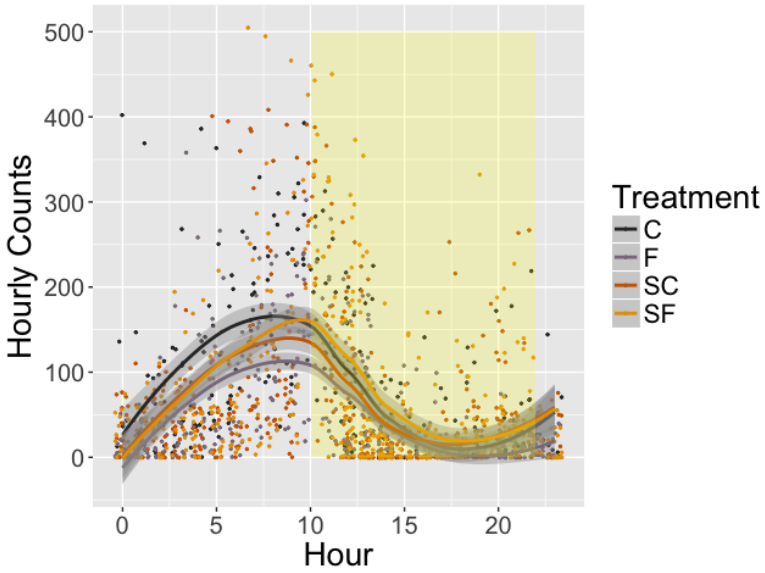


FIGURE 1.10: The hourly activity over 24 hours for *Drosophila* exposed to different cues from either crickets (C), "stimuli" flies (F), spiders fed crickets (SC) and spiders fed flies (SF). The yellow region shows times of light, and the loess splines for each treatment have the 95% confidence interval around them (shaded region).

Locomotory Activity of Experimentally Evolved Populations

The populations of *Drosophila* evolved with different predation selection, experiencing either predation from mantids or spiders showed a significant reduction in the baseline activity counts each hour compared to the control population (evolved with no predation) (chisq= 6.52, p<0.04, Fig. 1.12). Compared with no predation selection populations, there is an observed reduction due to spider predation of

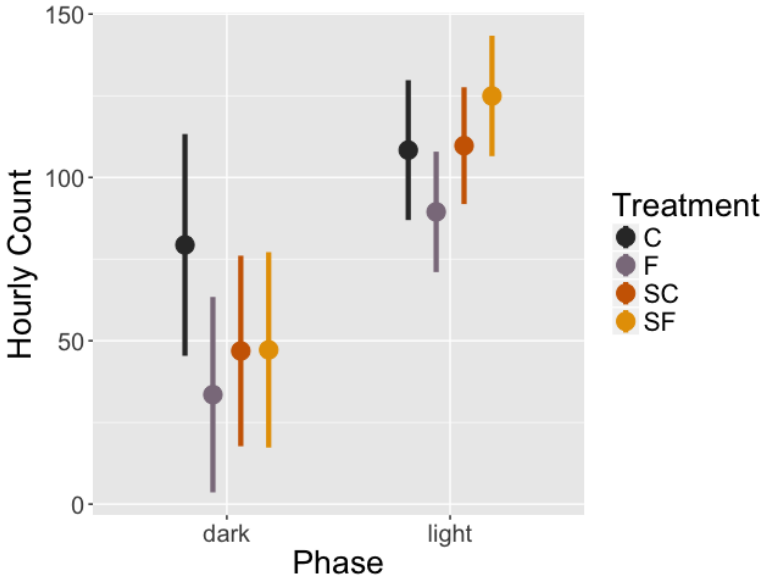


FIGURE 1.11: Four cues used for the comparison of light vs dark hourly activity (phase), either crickets (C), "stimuli" flies (F), spiders fed crickets (SC) and spiders fed flies (SF). Error bars represent 95% confidence interval.

-50.82 ± 23.96 and a reduction of -57.57 ± 23.96 due to mantid predation selection. There appears to be a significant interaction between treatments and light phases (chisq=7.42 , p< 0.03, Fig. 1.13), with the spiders increasing activity due to light from the mean by 21.99 (± 8.71) and mantids increasing by an estimated 18.73 (± 8.71) compared to the no predator treatment.

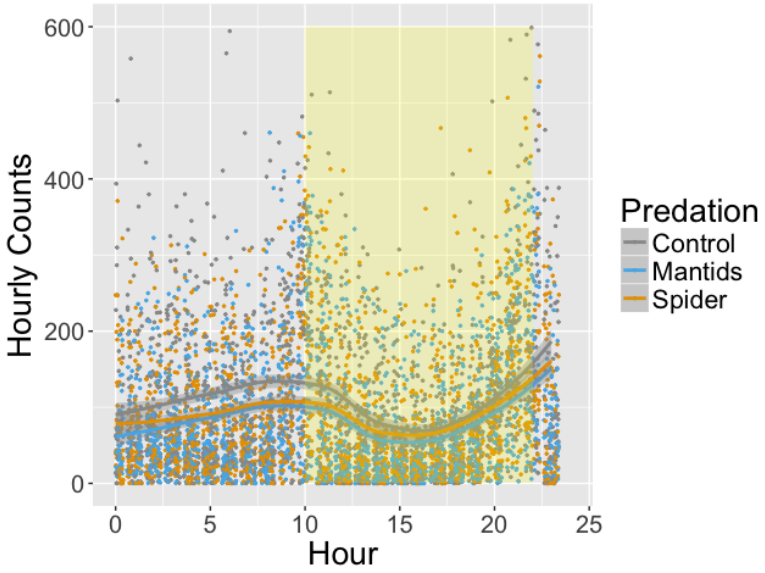


FIGURE 1.12: The baseline hourly activity over 24 hours for 12 populations of experimentally evolved populations of *Drosophila*, with 4 replicated of each treatment. Selection treatments had populations experiencing either no selection (controls), selection from zebra jumping spiders (spiders) or selection from Chinese praying mantids (mantids). Each treatment has an associated spline, fitting the data to a best fit line of daily activity trends, each of which has a 95% confidence level around the line (shaded region). The yellow region indicates the flies were experiencing lights being on.

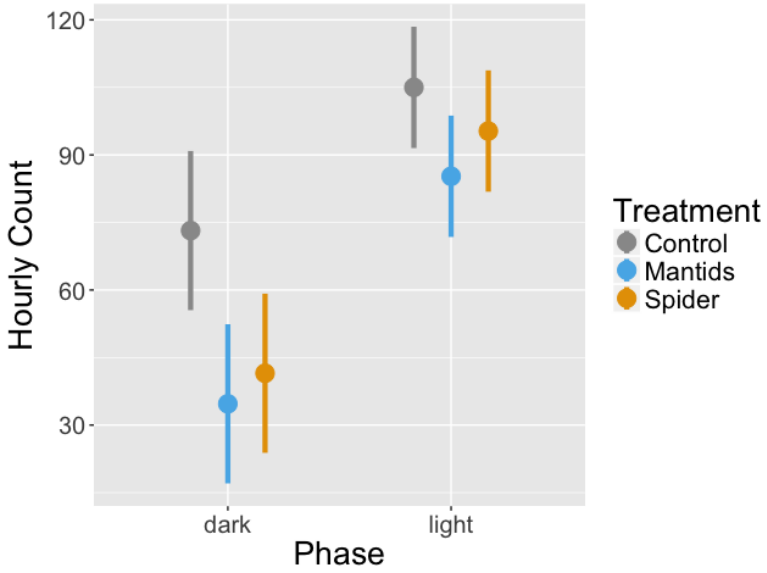


FIGURE 1.13: The hourly activity of *Drosophila* during 12 hours in light and 12 hours in darkness for the predatory evolved populations of no predation controls, spider predation selection and mantid predation selection. Error bars represent the 95% confidence interval.

1.4.3 Predation does not appear to elicit any Plastic or Evolved Response in *Drosophila* Mating Behaviours

Courtship Behaviours of Wild Caught Population

The presence of a predator slightly reduced the proportion of time a male *Drosophila* courted females ~3% ± 5%, but this variation was non-significant (N=64, chisq = 0.39, p= 0.53, Fig. 1.14). The number of courtship bouts in 15 minutes had an estimated difference of 0.52 ± 1.17, but this was non-significant (N=64, chisq = 0.19, p=0.66, Fig. 1.15). As expected using immature females, there was no recorded copulation during the 15 minute observations for the experiments investigating male courtship.

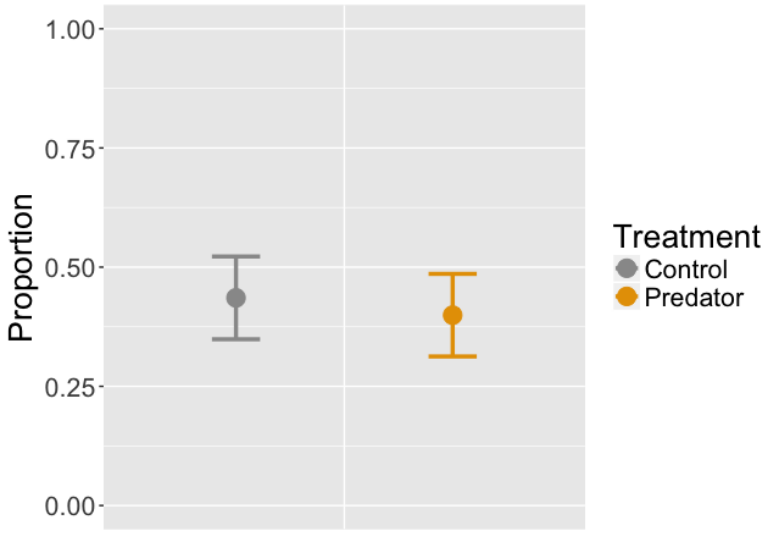


FIGURE 1.14: The proportion of time a male *Drosophila* spent courting a female in 15 minutes of recording for flies exposed to either a zebra jumping spider predator or no predator controls. Error bars represent the 95% confidence interval.

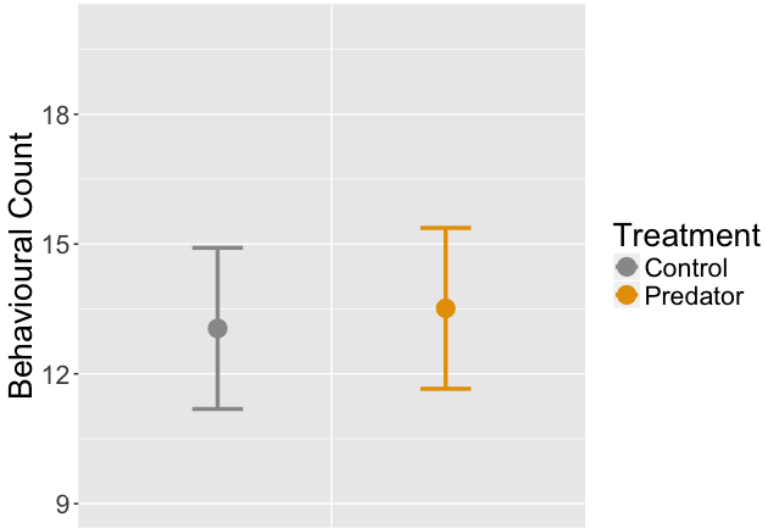


FIGURE 1.15: Number of independent courtship bouts a male *Drosophila* attempts in 15 minutes toward a female when exposed to either a predator (spider) or control (no predator), with 95% confidence intervals as error bars

Copulation Behaviours of Wild Caught Population

The number of successful copulations by males was found to not vary between treatments with spiders (predator) or without spiders (control) ($N_{control}$ = 72, $N_{predator}$ = 70, chisq = 0.022, p=0.88, estimate = -5% ± 33%, Fig. 1.16). For those that did achieve copulations ($N_{control}$ = 41, $N_{predator}$ = 39), a non-significant difference in relative copulation latency (50.63 seconds ± 91.99, chisq = 0.31, p=0.58, Fig. 1.17) and copulation duration (58.27 seconds ± 36.04, chisq = 2.61, p=0.11, Fig. 1.18) was observed.

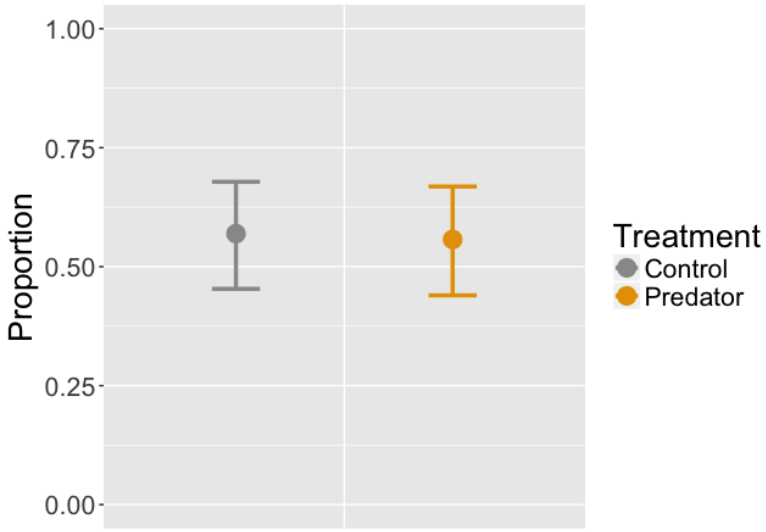


FIGURE 1.16: Proportion of successful copulations of a male and female mating pair of *Drosophila* in 30 minutes with either a spider or no spider present (predator vs. control). Error bars are the 95% confidence interval.

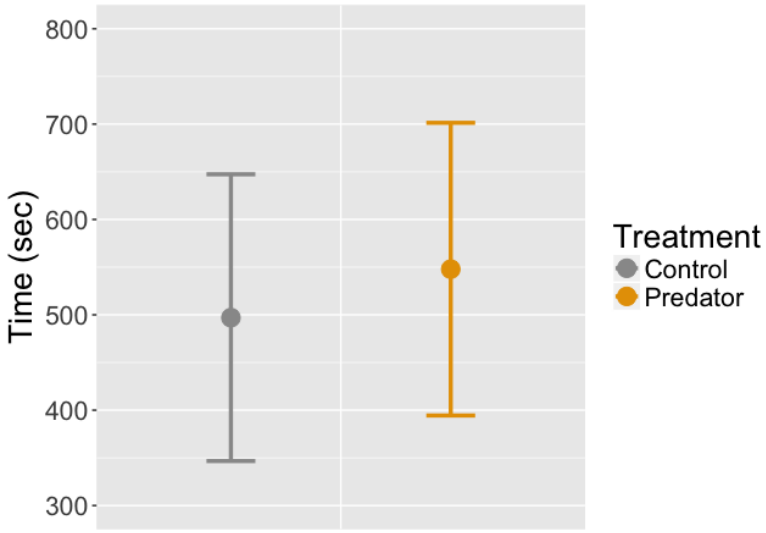


FIGURE 1.17: *Drosophila* mating pair time (seconds) to begin copulating after introduction when exposed to either a spider predator or no predator controls. Error bars represent the 95% confidence interval.

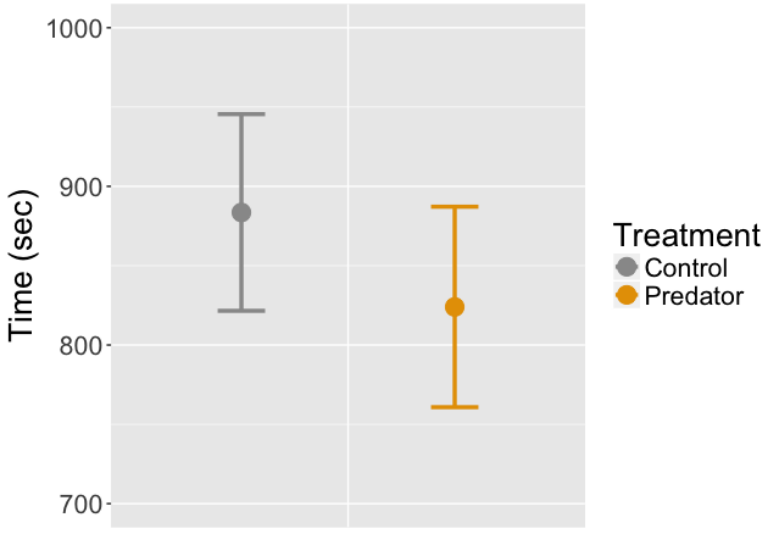


FIGURE 1.18: Length of a copulation bout (seconds) between a male and female *Drosophila* when exposed to a predator or without a predator, with 95% confidence

Evolved Population Mating Behaviours

For the evolved populations of flies, the measure of courtship latency (first instance of courtship observed) showed no significant differences between selection imposed on the populations (Fig. 1.19, chisq = 0.9, p=0.64). Courtship latency did have significant variation based on age (chisq = 14.59, $p < 0.003$), with reduction in age bins 2,3 and 4 from the mean of age bin 1 (age bin 2; -803 ± 381, age bin 3; -424 ± 400, and age bin 4; -900 ± 412). Within each age category, a varied response was seen, but each shows a non-significant change. Age bin 1 (1-3 days old) had approximately a 170 second decrease for both predation treatments compared to the control (-160 ± 380 for mantids /-180 ± 390 for spiders). Age bin 2 (4–7 days), 3 (8–11 days) and 4 (12–15 days) each showed a similar response (mantid/spider; +470 ± 540 / +630 ± 540, -260 ± 550 / +170 ± 560, -80 ± 570 / +140 ± 570 respectively).

Similar results were observed for copulation latency (Fig. 1.20), with no significant difference observed (chisq = 2.51, p = 0.28) between treatments. Once again age bin had a significant effect (chisq = 81.18, $p < 0.0001$), with age bins 2–4 reducing time to copulation by a large degree (estimated between 2000-3600 seconds for other age bins ± ~800) compared to age bin 1 estimate. There is also a non-significant change within age groups and treatment interaction compared to the control, with mantid populations generally decreased latency for copulation start for all age bins, except age bin 4 (1; -300 ± 900, 2;-300 ± 960, 3; -35 ± 960 and 4; +300 ± 970). Spider populations showed a wide range of responses for age bins (1; -900 ± 920, 2; +200 ± 1010, 3; +690 ± 1010 and 4; +990 ± 1020).

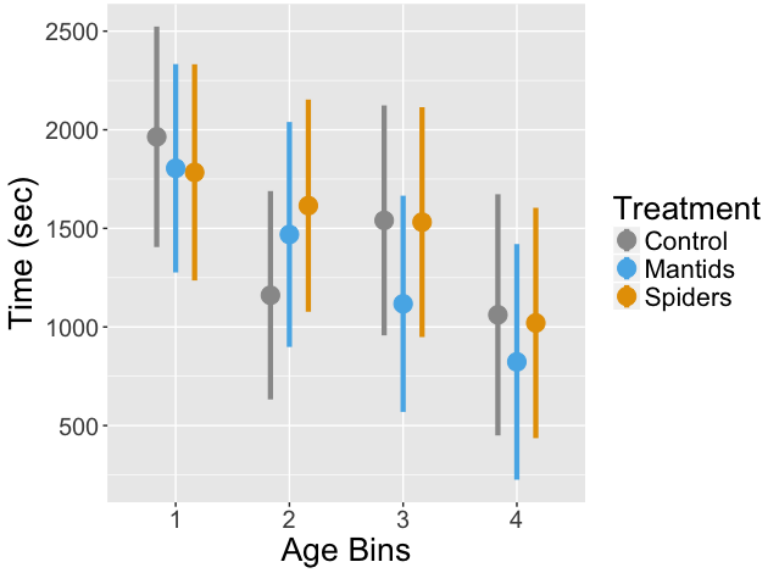


FIGURE 1.19: Time (seconds) to first observed act of courtship in a mating pair of *Drosophila* from three selection treatments: no predation, mantid selection or spider selection, with error bars representing 95% confidence intervals. Age bins correspond to range of ages, with 1 = 1–3 days old, 2 = 4–7 days, 3 = 8–11 days and 4 = 12–15 days

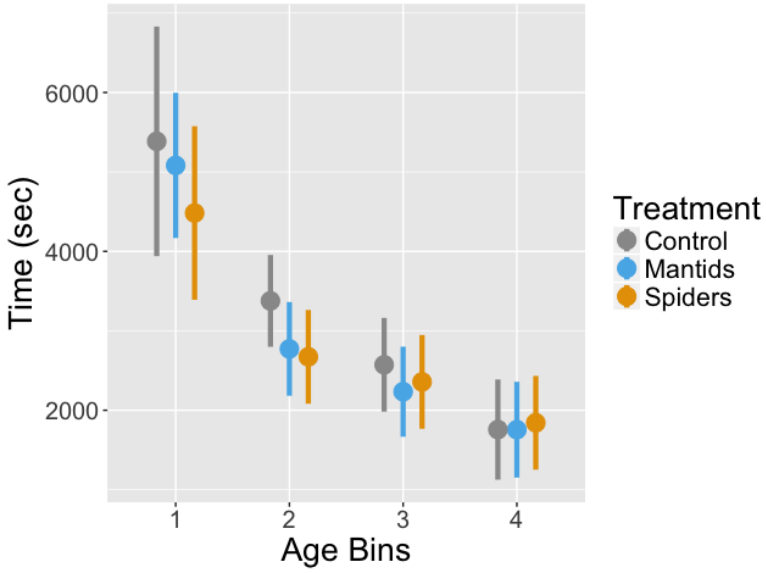


FIGURE 1.20: Time to copulation initiation (seconds) for mating pairs of *Drosophila* in age bins corresponding to range of ages (1 = 1–3 days old, 2 = 4–7 days, 3 = 8–11 days and 4 = 12–15 days). Each treatment corresponds to one of three selection treatments, no predation controls, mantid predation or spider predation, with error bars representing 95% confidence intervals.

No significant changes in evolved copulation duration (Fig. 1.21) was found for these populations= (chisq = 0.0045, p=0.998), with estimated effects of selection regimes (mantids and spiders) and age bins (1–4) having no large effect compared to control estimate (mantids: 1; +170 ± 180, 2; -130 ± 200, 3; -180 ± 200 and 4; -250 ± 200, spiders: 1; +60 ± 200, 2; -30 ± 200, 3; -50 ± 210 and 4; -100 ± 210). Age bins had a significant effect on duration of copulation (chisq = 13.59, p < 0.004), with each age bin from 2 to 4 increasing duration more as the flies got older (+64.53, +187.47 and 294.44, all ± ~165).

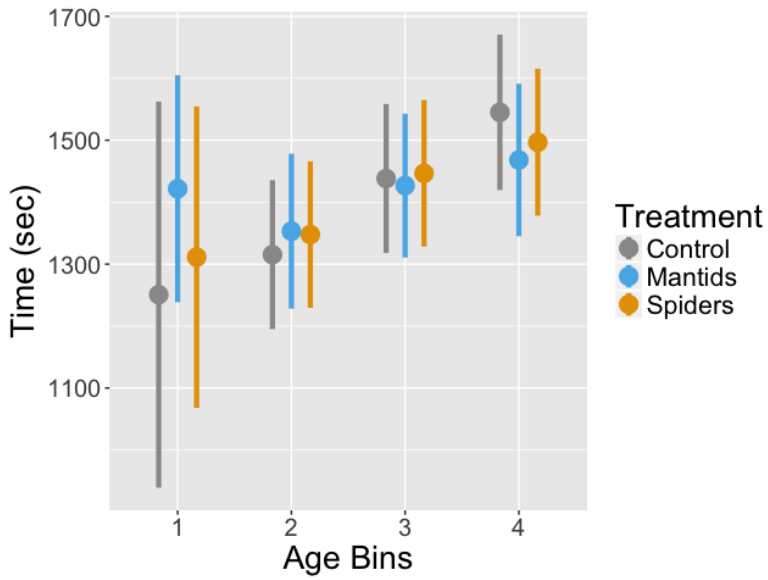


FIGURE 1.21: For 4 different age categories (1 = 1–3 days old, 2 = 4–7 days, 3 = 8–11 days and 4 = 12–15 days), the duration (seconds) of a mating pair's copulation bout in *Drosophila* is recorded for populations undergoing either spider predation selection, mantid predation selection or no predation selection (controls). Error bars are 95% confidence interval.

Copulation count (Fig. 1.21) was found to have a non-significant difference between treatments (chisq=1.103, p=0.58), but did have a significant difference based on age (chisq = 79.67, p < 0.00001). No significant variation between treatments

within age ranges was observed (chisq = 4.41, p = 0.62) for mantids (1; +1.37 ± 0.733, 2; -1.88 ± 0.985, 3; -0.779 ± 1.074 and 4; -1.33 ± 1.23) or spiders (1; +0.594 ± 0.785, 2; -0.328 ± 1.073, 3; -0.328 ± 1.073 and 4; +23.8 ± 13270). Note for age bin 4 for spiders populations, the estimate and range is large due to 100% success rate for copulations for the mating pairs sampled. This is believed to be due to the issues of complete separation, where the treatment/age bin (predictor variable) perfectly predicted the outcome variable (copulations), such that the model will result in an error. The model still gave estimates and was used to calculate the chi-square/p-values (shown above), and these results matched results to a simpler model (glm) to justify the inclusion here (results not shown).

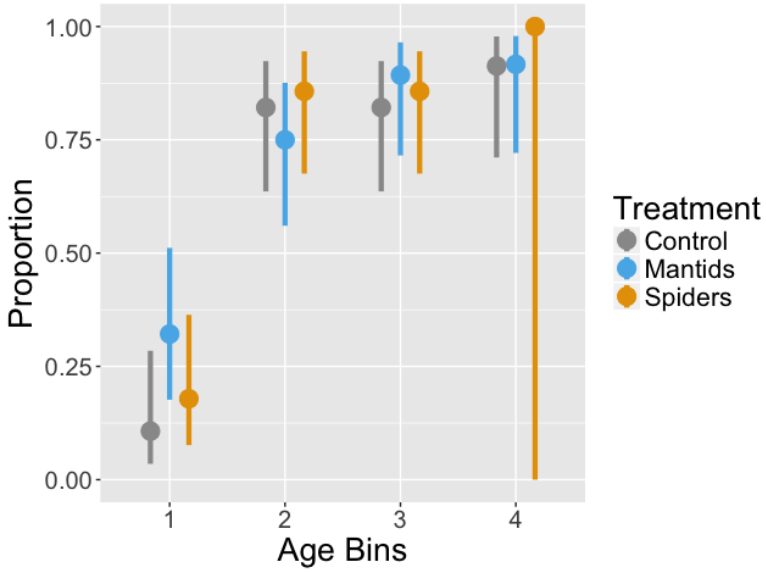


FIGURE 1.22: The proportion of individuals found to achieve copulations after 3 hours of recording time for populations of evolved flies with no predatory selection, or selection from either spiders or mantids. Age bins correspond to an age range (1 = 1–3 days old, 2 = 4–7 days, 3 = 8–11 days and 4 = 12–15 days) and error bars represent the 95% confidence interval. Age bin 4 large error bar is possibly due to complete separation issue, where the predictor (treatment/age bin) results in perfect prediction of the outcome variable (i.e. 100% copulation success), see text for details.

Courtship Times of Evolved Populations in Light Compared to Dark

Although there is a significant increase in the proportion of mating times in the light vs. dark (+32% ± 5%, $p < 0.00001$) as expected, no significant difference was found between selection treatments (chisq = 3.09, p = 0.21) or treatment by light interaction (chisq = 1.75, p = 0.42), with estimated variation in the dark (spiders; +2% ± 5%, mantids; -0.1% ± 5%) and light times (spiders; -2% ± 7%, mantids; -8% ± 7%) showing little variation (Fig. 1.21). Observer showed some effect, with observer R showing a decrease (-7% ± 3%) compared to observer A (chisq = 6.45, p <0.02).

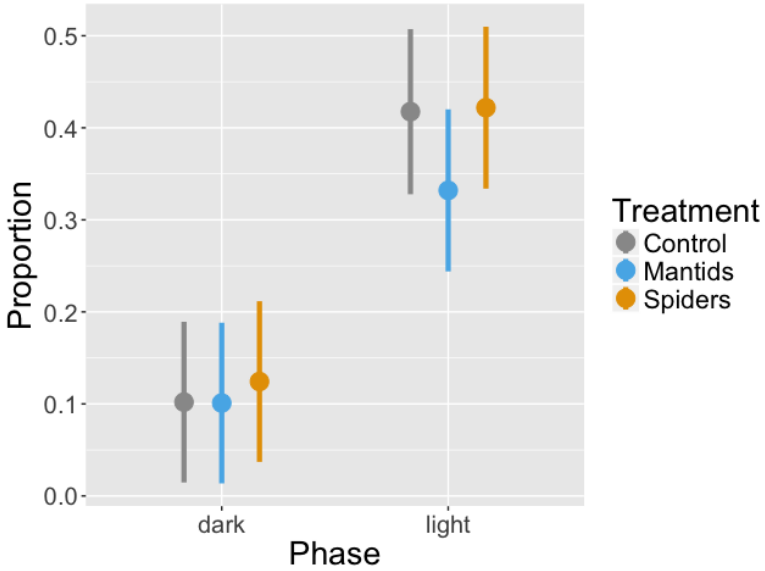


FIGURE 1.23: The proportion of time courting in 15 minutes during the dark or light phase for evolved populations with either mantid predation selection, spider predation selection and no selection (control).

1.5 Discussion

Through examination of three distinct aspects of behaviour (predation detection/avoidance, locomotor activity and mating behaviours), I have found mixed results for how predation elicits behavioural responses (either plastic or evolved) in *Drosophila melanogaster*. The expectation was that under high perceived risk, a wild caught population of flies and genetically variable DGRP dihybrid lineages would lower investment in traits thought to be risky (i.e. mating, activity), as well as avoid predator locations (Lima and Bednekoff 1999). However, this was generally not seen throughout the assays. Some support was found for flies to reduce activity when encountering spider olfactory cues compared to no cue controls, as well as evidence for genetic variability in predator avoidance. However, the overall trends of mating behaviours, population level avoidance, and activity with more complex cues did not support my predictions.

The expectation for populations exposed to predators for a long frequency of time was that evolution would favour those that are able to modulate risky behaviours to levels more beneficial to lifetime fitness. It is surprising to see no change with mating behaviours in the evolved populations, while there is change for locomotor activity. The encounters *Drosophila melanogaster* has with predators in obviously a more complex interaction that simply stated by the "risk allocation hypothesis" and the extended predictions to the evolution under risk.

For predatory avoidance, I have found evidence for genetic variability in *Drosophila* females (Fig. 1.1) and males (Fig. 1.2) in regards to spider avoidance, with this variability ranging much greater than expected, seeming to have lineages preferentially

going to spider cues. Overall, the population trend of all DGRP lineages together had approximately 50% predator avoidance, indicating that there is no strong population propensity for predator avoidance. Oddly as well, there was a weak negative correlation between male and female avoidance within DGRP lineages (Fig. 1.3) when a positive correlation would be expected for each DGRP line.

When comparing the plastic response to locomotor activity, different cues have complex effects on activity. With mantid cues (Fig. 1.4) *Drosophila melanogaster* males showed no evidence to alter active behaviours (as seen in other studies; Elliot *et al.* 2017, Parigi *et al.* 2014), and spider olfactory cues showed some evidence points to reduce *Drosophila* locomotor activity (Fig. 1.6) compared to no olfactory cues. Within the selection populations, there is clear evidence that the predation from both spiders and mantids is causing strong selection for a reduction in activity levels compared to predator naive populations (no risk) (Fig. 1.12). The activity reduction with spiders cues and within the evolved populations are important because they can be contrasted with the mating assays. We see that although activity may be affected by predation, across all measures of mating behaviours completed, I have found no significant effects for both plastic and evolved response in mating behaviours (Figures 1.14 – 1.23), indicating predation may not play a major role in *Drosophila* mating decisions.

As predator avoidance is expected to be an evolutionary beneficial trait, especially when a predator and prey first encounter each other (Lima and Dill 1990), I had anticipated that flies would be able to perceive predatory threat, and avoid areas with predators. However, as a collective, the DGRP lineages appear to not avoid spider cues, due either to a lack of perception to threat (visual, olfactory or

otherwise) or out of choice. This is odd when looking at the variation between DGRP lines as there appears to be genetic variability for sex specific line effects (Fig. 1.1 and Fig. 1.2). However, this variation does not follow the predicted patterns that was assumed to be predominant for flies. I had expected correlated patterns of avoidance within DGRP lines between males and females, as well as the genetic variation in avoidance to fall somewhere between random assortment (50:50 split) to high avoidance. However, sex showed an overall weak negative correlation between lineages (Fig. 1.3) and some DGRP lineages appearing to show variation to preferentially go to spiders.

It is unclear whether this is a product of the experimental design or due to the flies themselves not showing any propensity for predator avoidance. As there was found to be a significant effect of the vial location with the spider (1.4.1), the apparent random assortment of flies may be a product of the layout of containers. As each line was randomly placed within containers, and on each day there is 50% of vials with spiders in the front vial, and 50% with the opposite layout, the population mean sitting at 50% avoidance may be a product of this design. As there appears to be preferences to the front vials, the assortment for the full population would tend to be based less on spider, but the location of the vial. Although this may explain some lines showing preferential choice to spider vials, the methodology was completely random, and the genetic variability around this mean should still be indicative of the overall genetic variation detected. Although row showed an effect, this was found to be within row 2 and 4, and not between 1 and 3, which is odd that there is no patterns of differences further from the front row. It may be other explanations to all these odd results.

As this assay is a simple choice test, many additional behaviours may be playing a role, cues may be overwhelmingly strong within the containers that flies cannot distinguish "safe" vials or that the vials themselves may be difficult to see predators through. These may impact either the olfactory cues or visual cues *Drosophila* can distinguish, processes that may be important in predator detection (de la Flor *et al.* 2017, Ebrahim *et al.* 2015).

Another possibility may be that some of the 60 DGRP lineages used have an odd suite of behaviours or impacted sensory structures that can alter detection or avoidance. The one common element between all lines was the use of the "tester" line (DGRP line 83) to create DGRP dihybrids to dampen the costs of inbreeding depression (Zwarts *et al.* 2015). It may be that this "tester" line is present with traits that would alter the perception and avoidance of predators, or be present with traits incompatible with other lines used. It may instead be possible some lines used (particularly those with odd sex differences and preferential movement to spiders) may be the product of these lineages having a suite of traits that attract them to spider vials, make spiders undetectable or many other possible reasons to go toward predation threat.

Studies have shown honeybee prey (*Apis mellifera*) are attracted to flowers with predator crab spiders (*Thomisus spectabilis*) (Heiling *et al.* 2003). This is believed to be the product of spider UV reflection of attractive flower patterning for honeybees, as well as predators choosing more attractive flowers (Heiling *et al.* 2003, Heiling *et al.* 2005). Other predators have used techniques to lure prey to themselves, such as flower mimicry in orchid mantid (*Hymenopus coronatus*, O'Hanlon *et al.* 2014) or the tongue of the alligator snapping turtle (*Macroclemys*

temmincki) appearing as a wriggling worm (Spindel *et al.* 1987). It may be that the odd behaving lines are not able to distinguish between a risk and potential predator deception to find a safe food patch, a skill other lines are expressing.

With some genetic lineages seemingly going to spider cues, it would be expected that within the predator selection populations used elsewhere, this "spider preference" would be selected out of the population. This would have been a nice secondary prediction to test, expecting evolved predation populations to show high predator avoidance, selection upon "predatory avoidance genes" and removing "predatory preference genes". However, this could not be tested, as some time after these populations changed locations from Michigan State University to McMaster University, many of the populations succumbed to an antibiotic resistant bacterial infection on the food media that limited larval survival. This resulted in many extinction events of populations, and any that manages to recover had been dealt major population bottlenecks, inadvertent selection through many tested antibiotics, and long stretches of relaxed predation selection to allow flies to recover. This ultimately lead to these populations not being a viable study group for an additional experimentally evolved traits. Future experiments that create populations of experimentally evolved populations can build on this idea though, and examine if populations can be selected for high predator avoidance, and possibly (if ambitious with a carefully planned design), select individuals that prefer to be close to predators, supporting the idea that the variability can stretch to both extremes.

For locomotor activity, there appears to be an evolved reduction in the activity levels under selection from both mantids and spiders compared to those evolving

without predators (Fig. 1.12). This is interesting as *Drosophila* have a reduced activity with direct predator olfactory cues due to spider cues (Fig. 1.6), but not with mantid cues (Fig. 1.4). These findings for direct mantid cues are in line with the experiments done by Parigi *et al.* (2014) and Elliot *et al.* (2017). Here, there was no recorded alteration in activity with only mantid olfactory cues, and these previous experiments found no effect of direct predatory exposure, which gives confidence that mantid predation does not result in a locomotor activity plastic response in *Drosophila*. Interestingly, with the reduction in activity in the mantid selected evolved populations, mantids appear to be a threat to *Drosophila* and selecting for a less active population. As this reduction is not seen with direct exposure of the recently caught population, it seems that flies are not recognizing mantids as a threat and it is only after years of selection will flies alter behaviours in response to mantids.

While the mantid olfactory cues (and other studies with direct cues) showed no alterations in activity of *Drosophila*, measuring activity with spider olfactory cues showed locomotor activity adjustments for wild caught *Drosophila*, specifically depending on whether spider cues were present or absent (Fig. 1.6). However, no differences were observed due to cricket cues compared to spider cues (Fig. 1.8), suggesting any significant adjustments seen (i.e. when compared to no cue treatment) may be due to a new and unfamiliar olfactory cue (neophobia), and not due to a plastic response to predators. Additionally, the comparison between the 4 sets of alternative cues (crickets, spiders fed flies, spiders fed crickets, and con-specific "stimuli" *Drosophila*) causes some confusion with this result, as treatment was found to be significantly different, with reductions in overall activity for the spider

treatments and the "stimuli" fly treatments compared to the cricket treatment (Fig. 1.10).

Just looking at the two spider treatments, they appear to be causing flies to behave very similarly, indicating the diet of spiders did not alter *Drosophila* activity. The cues from con-specific *Drosophila* males showed a reduction in *Drosophila* activity, which may be due to the three male flies used to elicit the cues. As these males may be engaging in aggression to one another they may be releasing cues expressing stress or injury. These cues can effect the behaviours of con-specifics as in two *Etheostoma* species, (Smith 1979), a pattern seen in many other (generally aquatic) species (review by Ferrari *et al.* 2010).

However, what is most interesting to look at is the response between *Drosophila* adjustments in activity with cricket cues, spider cues, and no cues. When comparing the spiders to the crickets from the 4 way comparison experiment, the results are more similar to the spider cues vs. no cues experiment (Fig. 1.6), with spider cues eliciting a reduction in hourly activity. However, this is not similar to the comparison of cricket cues vs spider cues (Fig. 1.8), which (although non-significant to alpha 0.05), was trending to a larger reduction due to cricket cues rather than spider cues.

With all the results together, the response of *Drosophila* is quite surprising. There is some support for a neophobic response between spider predators and non-predator crickets, but it appears *Drosophila* do not respond to the potentially novel olfactory cues of mantids (Fig. 1.4). Due to the lack of variation with mantid cues on *Drosophila* activity, it may be that the neophobic evidence between

crickets and spider olfactory cues may be due to a generalized response to certain olfactory cues for spiders and other insects. It may be that *Drosophila* response to olfactory cues is a evolved antipredator response that reacts to a suite of spider predators (*Arachnida*) and a possible suite of the family *Insecta* (including crickets; *Orthoptera*), but not detect cues from the more recently introduced Chinese praying mantids (introduced about 200 years ago). This idea was partially supported by personal observations of few interactions of mantids and *Drosophila* in natural settings by Parigi *et al.* (2014), limiting *Drosophila* natural evolution to respond to mantid olfactory cues.

To be more sure if this is neophobia to a possible suite of insects and spiders or if spider cues are responsible for a decrease in activity when compared to cricket cues, an increased sample size throughout these assays should be done (currently between 14 and 16 thus far for each experiment treatment). If it is a neophobic response, follow up work with a) a non-predator arachnid and b) other cricket/ insect species should be done. This will allow for analysis if a similar active response will be displayed in *Drosophila* between all cues. If this trend continues, there is likely a generalized olfactory cue shared within *Drosophila* that is taken as a threat.

Both mantid and spider predation as a selective agent has resulted in a reduction in baseline locomotor activity. This is potentially the result of highly active flies at a higher risk of predation in the experimental cages, resulting in selection for an overall reduction in activity. This runs counter to the ideas suggested in Parigi *et al.* (2014), stating that due to the active hunting mode of spiders, there would be an increase in activity (which was seen by de la Flor *et al.* (2017) and Parigi

et al. (2014)), while ambush hunting species (i.e. mantids) would reduce activity (not seen by both Elliot *et al.* (2017) and Parigi *et al.* (2014)). However, this prediction (and associated results) were based on direct predator cues and threats to an individuals survival. When we observed spider predation cues directly we saw a reduction in the overall activity of flies, contrary to the results from de la Flor *et al.* (2017) and Parigi *et al.* (2014). As these experiments with spiders only used olfactory cues, the observed reduction from figure 1.6 may be due to the olfactory cues present not being a strong indicator of threat to *Drosophila*. By not being able to discern the type of threat, the *Drosophila* can possibly not respond correctly, which may support the idea that when a generalized olfactory cue is present, flies cannot distinguish between predators and similar species of non-lethal organisms. It is potentially important for both visual and olfactory cues to perceive a threat and modulate behaviours based on both cues.

Within the population cages, the ambush hunting mantids appear to select upon a reduced activity for both light and dark times, and spiders also appear to select upon a similar reduction. This may be due to the nature of the predators as visual hunters and our flies requiring vision as an important survival trait. During the night, both the flies and the predators are more visually impaired, and as such, flies may reduce activity to avoid encountering a stationary predator (spiders visually observed to make webbing at tops of cages and remain stationary). Upon analysis of predator activity, the spiders (B: Fig. A1.6) showed very little activity at night, with a drastic increase during the day. With the increased daytime activity of spiders, a reduction in activity during light times will be beneficial to not draw the attention from the visual predator (Freed 1984, Jackson and Pollard 1996).

The mantid predators did not exhibit any drastic activity shifts based on night or day (A: Fig. A1.6). This would favour a reduction in activity at all times to avoid running into mantids (night) or being detected by the mantids as they as well are visual hunters (Prete 1999).

Using the assumption that activity is an overall risky behaviour, we see that, although complex with alternative cues, direct spider cues a shown to reduce this predicted risky behaviour and both predators are selecting on a reduction in activity. Unlike these observations of locomotor activity, mating behaviours of *Drosophila* appear to have no plastic response to spider predation risk, or an evolved response from mantid or spider selection.

To assess *Drosophila's* plastic response in mating behaviours to predation threat, a recently caught population of flies was used that has experienced very little domestication to the lab. The expectation was that mating activities would put both members of a mating pair at increased risk, and thus, I expected flies to abandon mating and focus primarily on immediate fitness benefits (i.e. vigilance and survival). This was not seen however, as flies with or without predators present behaved similarly, such that the presence of a predator did not influence mating behavioural times (Fig. 1.14, Fig. 1.17, Fig. 1.18) , proportion of successful copulations (Fig. 1.16) or courtship behavioural counts (Fig. 1.15). Although not a direct prediction for this experiment, but similar to our predictions from our analysis of the evolved populations, predation did not promote earlier receptivity to mates, as our young, rejecting females did not accept copulations regardless of treatment. These results taken together indicate that spider predators are not altering mating behaviours in *Drosophila* in any way measured, neither promoting earlier or

quicker mating, nor changing male or female mating behaviours.

This is an odd result for this population of flies, as the behaviours involved with mating should put flies at a greater risk (Candolin 1998, Lima and Dill 1990). This is a somewhat similar response as in the dumpling squid (*Euprymna tasmanica*, Franklin *et al.* 2014), where predation showed little effect on the mating behaviours in males and females. The authors conclude that the squids may be prioritizing reproduction over survival. The theory was that the chance of future reproduction may be low for these squids, and any opportunity for reproduction should be taken. This may be true in *Drosophila melanogaster*, where the benefits to mating are greater than potential costs to predation risk. Due to the short lifespan of *Drosophila* species, any opportunity flies are able to achieve mating will be vitally important, such that the need for mating may outweigh the risks to surviving predatory exposure, as proposed by Franklin *et al.* (2014).

An important note that could be affecting the flies used for plastic responses to predation would is their sexual and social history. All male and female flies used are virgins with no sexual experience, and for the males, they have no social experience, being in isolation since eclosion. Isolation has been shown to effect aspects of *Drosophila* behaviours such as aggression (Hoffmann 1990), as well as lifespan (Ruan and Wu 2008). This isolation may possibly be the reason the males choose to engage in mating rather than antipredatory behaviours. The presence of the opposite sex for the first time may drive away any thoughts of survival, as this may be perceived as the only chance for any future fitness benefits. This may even be elevated when the risk is high, as both the initial contact with the opposite sex, and the risk to survival may drive mating to occur. As the short lifespan of

the *Drosophila* may normally drive mating over vigilance (as with Franklin *et al.* 2014), the isolated virgin males and virgin females may see the first chance with another mate as the only opportunity to gain a mate (and increase lifetime fitness) despite the risks.

It should also be noted that these flies are trapped in arenas with a predator present. In nature, there will be opportunities for escape before engaging in mating behaviours. Flies have been shown for high activity (believed to be exploration for escape) with jumping spiders initially, before reducing activity to more normal levels (de la Flor *et al.* 2017). As these flies may see no escape as possible, the mating pair may abandon antipredator behaviours and attempt a last ditch effort to gain mating. These flies have been shown to respond to spider cues (Fig. 1.6), so they should be aware of the risk that is present, but are not changing mating behaviours as a response to this risk.

As the mating displays between a mating pair involve both auditory (wing song), movement (following) and attention to a mate (orientation, licking, copulating), mating behaviours are expected to be risky for *Drosophila* with predators present (Hall 1994, Lasbleiz *et al.* 2006, Spieth 1974). Over many generations with predatory selection then, the time spent exhibiting these behaviours is expected to be reduced in order to gain matings at the least risk to both members of a mating pair. It would also be beneficial to engage in mating at a earlier age, in order to gain future fitness benefits before potential capture. As previously discussed, we have seen that selection is altering locomotor behaviours (Fig. 1.12), but this does not relate to is not seen in these populations mating behaviours.

The mating behaviours in the evolved populations were not observed with predators present, but rather in a no risk environment. As with measures of activity, the interest was the evolved behaviours that have been passed on through years due to selection and not the result of a response to direct predation. However, it appears selection did not favour any alterations in age of mating or mating behaviours measures. Although it should be beneficial in a setting with high predation risk to achieve mating earlier (both based on age and overall time exhibiting behaviours, Fig. 1.19 – Fig. 1.22) as well as the focus on mating in lower risk settings (i.e. night vs. day, Fig. 1.23), there is no observed changes for courtship and copulation times and proportions across treatments that evolved under predation risk as compared to the control lineages. Although observer showed to have some effect on recorded courtship, the complete lack of variation between populations would not be explained by variation in observers.

One possible explanation is that without the presence of a predator (low risk) along with the presence of the opposite sex, flies behave "normally". The flies may be seizing the opportunity to mate in this low risk time, which fits the model of the "risk allocation hypothesis" (Lima and Bednekoff 1999), to take advantage of low risk times to engage in risky behaviours. However, after years of selection with predators (with constant risk), the expectation would be that some shift in behaviours to be evolutionarily beneficial and certain favourable behavioural traits will be expressed, regardless of predation risk, which is seen with the activity results (Fig. 1.12). A simple explanation could also be that mating behaviours do not pose an increased risk to the mating pair. This seems an unlikely scenario as selection seems to favour those with lower activity, which is potentially linked with mating

behaviours, which require many movements by both males and females (Hall 1994, Spieth 1974).

This outcome may instead be a product of the high density of flies within the cage. Mating opportunities are high in the cages used, and the opportunities to increase future fitness is easily accessible with low risk due to lower predation risk with the large group size (Foster and Treherne 1981). As there is selection by predators, but many mates, there is opportunity to mate early in life and not worry about survival to the degree they may in nature, as investing in future fitness can be achieved earlier in life. However, we see no shifts for earlier ages (namely age bin 1) for more copulations, nor quicker courting and copulating between treatments within this age category. Instead, all treatments for age bin 1 (1–3 days old) appear to be significantly different from the other age bins, but this would be the exception in nature, with the younger flies showing, for example, a lower copulation proportion (Fig. 1.22), as they are only may only be moderately receptive at this age (Manning 1967). It may be that mating may be such an important biological process for flies that altering these behaviours and receptive ages may be no simple task through evolutionary time.

There is one comparison that would support the idea of our selection due to predations altering behaviours, namely that there appears to be a sharp increase in times of behaviours across all evolved populations compared to the freshly caught population. However, these comparisons will not be accurate as evidence for an evolved response. This is because the freshly caught population is different from that of the initial population for the evolved populations. Also, the evolved populations having been housed in a laboratory environment for approximately 7 years

which may lead to domesticated traits and alterations that is due to how the populations have been housed. As the increase in behavioural times is shared across populations, including the controls, this is indicating that this may be a product of lab domestication *per se* and not a product of predation risk.

Based on the "risk allocation hypothesis" (Lima and Bednekoff 1999), under direct predator cues (i.e. first exposure for flies), *Drosophila* should yield high antipredatory behaviours and low risky behaviours (believed to include activity and mating). However, this is only partially supported by the data collected, with locomotor activity showing some variation with different risks, but mating experiments no supporting this model. What the "risk allocation hypothesis" is missing to more fully encompass interactions between predators and prey is to look at the life history of the organisms studied.

Specifically for *Drosophila*, due to the short life span and few opportunities for mating, the benefits with mating may outweigh any potential risks associated with predatory exposure. The potentially risky behaviours that do not yield as important future fitness benefits as mating would then be expressed as a method to increase immediate fitness, which may be seen with the reduction in activity. Essentially any potential investment in highly important future fitness effects will be more beneficial than investment in immediate fitness, as the low lifespan may allow for a low chance for any future reproduction. This is reflected with our evolved populations, as there appears to be no selection to alter mating behaviours, and flies (although at an increased risk) have not been selected to reduce investments in future fitness effects. For future analysis of predator-prey interactions, the natural history of the organisms in question will allow for a more comprehensive analysis

of these encounters.

With *Drosophila melanogaster*, one of the most commonly studied organisms, encounters with predators appears to be a complex interaction. It is not a simple cut and dry equation of increased predation equating to reductions in risky behaviours. For these experiments, I have found a suite of odd results that did not confer with many of the expected outcomes. *Drosophila melanogaster* is an important experimental organism, but has not been used for many experiments regarding predation. If *Drosophila* is to be used for further predation experiments, careful planning of the experimental design is needed, as behavioural modifications are complex and not intuitively obvious.

Chapter 2

Genomic Evolution of *Drosophila* due to Selection by Predation

2.1 Introduction

One of the most potentially influential and pervasive pressures that has shaped the adaptations of many organisms is predation acting as an agent of selection (Bengtson 2002). Nearly every organism is subjected to predatory selection, which can not only affect prey survival, but can have an associated loss of other fitness benefits as well. There are many examples of selected antipredatory adaptations, such as defensive structures (Hoso and Hori 2008, Palmer 1985), camouflage techniques (Stuart-Fox *et al.* 2008), or poisons and warning colourations (Myers *et al.* 1978, Williams *et al.* 2012). These adaptations have been selected upon to confer a survival advantage when encountering a predator. At the same time, predator encounters an have indirect effects on prey, such as lost mating or foraging opportunities. Predation not only selects upon those with adaptations to survive, but

those able to limit these indirect fitness effects, known as non-consumptive effects (Creel and Christianson 2008, Lima 1998). These non-consumptive effects are detrimental to the lifetime fitness of the prey and evolution will favour those able to lessen both consumptive (predation) and non-consumptive (lost future fitness opportunities) effects.

Evolution, in the broadest sense, enables alleles that boost an individual's fitness traits to increase in frequency within a population as alleles detrimental to fitness are being selected out of a population (Burt 1995, Darwin 1859, Orr 2009). Although there are many factors associated with the evolution of species, evolved traits can usually be classified as either naturally selected (selected to increase immediate fitness (i.e. survival)) or sexually selected (beneficially selected traits for future fitness in the form of mating) (Andersson 1994, Darwin 1871). Together, these fitness effects evolved populations which can allow organisms to reach optimal fitness peaks, creating the most fit population (Fisher 1958, Lande 1979, Lima and Dill 1990, Orr 2009).

I am interested in the evolution of beneficial alleles to selection by predators, and how predation can shape the genomes of prey. I am specifically looking at how allele frequencies change due to natural variation already present in the genome in populations experiencing high predation risk. Seeing how populations have changed through evolutionary time due to selective pressure can be difficult, as it is rare to know the starting state for the population under selection (i.e. the ancestral genome) and it is difficult to accurately predict the changing selection pressures that have shaped a population. Some studies can find a measure of the ancestral genome and find populations with predictable predation patterns, such as

with *Daphnia magna*. These organisms are found within man-made ponds stocked with a controlled number of fish, and researchers could predict the genetic differentiation within this population from the reconstructed ancestral state through stored "seed banks" (Cousyn *et al.* 2001). However, opportunities for experiments like this are rare, and can lack the control that is available when using laboratory experimentally evolved populations.

Experimentally evolving populations to laboratory selection regimes allows for both controlled patterns of selection on populations while being able to replicate the selection on distinct populations. This method has been common practice for inferring the evolution of species able to be raised within the lab at large population numbers with short generational times, such as in *Escherichia coli* (Elena and Lenski 2003, Lenski 2017), yeast (Parts *et al.* 2011) and within *Drosophila* (Orozco-terWengel *et al.* 2012, Turner *et al.* 2011). Now that sequencing technologies are more readily available with a reduced cost, the genomic evolution of experimentally evolved species can be more thoroughly studied. With inferences of ancestral genomic structures compared to the derived state of populations, how selection has altered genomes can be studied. These evolve and resequence (E&R; Turner *et al.* 2011) studies have been used to map associated genes with domestication (Rubin *et al.* 2010), trace the trajectories of alleles over generations (Orozco-terWengel *et al.* 2012, Tobler *et al.* 2014), and analyze variations due to body sizes (Turner *et al.* 2011), development time (Burke *et al.* 2010) and courtship songs (Turner and Miller 2012), all recent with the advances in sequencing technologies (Schlötterer *et al.* 2014). Using selecting populations of *Drosophila* with high predation risk, the evolution due to predator selection can be analyzed in a controlled environment

with repeatability and consistency. As well, by sequencing many generations, the patterns of allele frequency changes can be compared to the ancestral state.

Drosophila melanogaster (*Drosophila* or simply flies) is a common organism used for E&R studies, a species used throughout the fields of genomics, development and animal behaviour (Dietrich *et al.* 2014, Roberts 2006). Due to *D. melanogaster's* short generation time (new generations about every three weeks), and well defined genomic sequence (accurate annotated genomes available for sequence analysis), flies are a useful organism to study the evolution of beneficial alleles under controlled selection experiments (Burke *et al.* 2010, Mackay *et al.* 2012, Schlötterer *et al.* 2015). The *Drosophila* populations I used experienced selection from "episodic" bouts of predation by a generalist predator to *Drosophila*, the Chinese praying mantids (*Tenodera aridifolia sinensis*). These bouts of predation allowed surviving individuals of a high predation environment to pass on any beneficial alleles which potentially contributed to the survival of a bout of predation. Each generation, the populations experienced high predator mortality (approximately 40%) before being allowed the opportunity to lay eggs for the next generation. The expectation is that regions of the genome that confer a survival advantage will be selected upon, and beneficial alleles will increase in frequency within the populations.

As these populations were initiated from a genetically variable base population (Fig. 2.1), evolution will only be able to select upon alleles that are already present in the population. This experimental design allows for selection to act independently and randomly within each separate population replicate, selecting on any number of traits not predetermined previously to experimentation. That

is, selection can occur on these populations with little experimental interference. By using a strong selection regime, beneficial or detrimental traits of interest will independently be increasing or decreasing within separate replicate population. Evolution can then act randomly within each replicate population, with important regions for the specific selection regime hopefully expressed within both replicates. By comparing consistent genetic changes detected between selection replicates, highly beneficial regions have independently evolved in replicates through convergent evolved.

With that said, although selection is able to act "naturally" in that there is no researcher interference for particular traits, experimentally evolved populations are reared within the laboratory and are experiencing lab domestication. To separate inadvertent selection on *Drosophila* from being raised within the lab, it is important to have control populations; population replicates that experience the same conditions as selection populations but without the chosen selection pressues (i.e. predation). This allows separation of variations that have come about due to time reared under the novel laboratory environment, and variation due to the selection regimes (Orozco-terWengel *et al.* 2012, Schlötterer *et al.* 2015).

To avoid population bottlenecks, genetic drift and to allow for the highest probability for random mating, a large population size was needed (Schlötterer *et al.* 2015). *Drosophila* are able to be reared in large populations which can allow for the most "natural" selection to occur, with minimal evolutionary constraints. With a large population size, looking at population trends across the genome would require the sequencing of many individual flies, a powerful method, but ultimately costly and labour intensive. A similar method that can be used that is more

cost-effective while still producing comparable results is sequencing a pool of individuals as one. This method, known as pooled sequencing (Pool-Seq) has been shown to be comparable to individual sequencing for predicting population genetic variability and evolution (Gautier *et al.* 2013, Rellstab *et al.* 2013, Schlötterer *et al.* 2014).

By using different generational time points (pools of individuals at different generations) I want to examine how allelic variations changes over time and the evolutionary trajectories that predatory selection can have on a population of flies (similar to Orozco-terWengel *et al.* 2012). Specifically here, I present data on the selected sites within *Drosophila* under high predatory exposure to see the shifts in the genetic structures of *Drosophila*. The populations used have shown that there has been selection acting upon flies, specifically for wing shapes and survival when exposed to predators (DeNieu 2014, unpublished lab data), but what is unclear is the genetic underpinnings that are guiding the evolution of these populations. The overall aim is to see single nucleotide polymorphisms (SNPs) that are shared within predation populations but significantly different from the control populations across the genome. This research is ongoing, with the variation across the (almost) full genome presented. I will present the work up to this point, with the methods covering the extent of the data analyzed.

2.2 Methods

2.2.1 Chinese Praying Mantid Predators

For predatory selection, 1^{st} instar Chinese praying mantids (*Tenodera aridifolia sinensis*) were used, with 2^{nd} instar occasionally used depending on 1^{st} instar availability. Mantid egg cases, that can hold ~100-400 mantids, were collected in Southern Michigan and ordered from Nature's Control (Oregon). Egg cases were held in stasis at 4°C until set up for hatching fresh mantids, where the egg cases were moved to 24°C, 60% humidity. After eclosion, the group was split such that five mantids were held within a 710 mL cup with a side mesh window for air flow, a moisture tissue for humidity and an artificial plant, stored at 18°C, 60% humidity, on a 12:12 light:dark cycle.

2.2.2 Episodic Populations

These populations were initiated, maintained and selected at Michigan State University (MSU) with work done by Dr. Michael DeNieu, Mauricio Losilla and other previous members of the Dworkin lab at MSU. To initiate selection/control lineages, a variable base population was created with an advanced intercross (3 generations) of 100 inbred lines of *Drosophila melanogaster* that were collected from North Carolina and Maine (Goering *et al.* 2009, Reed *et al.* 2010). After many generations of random mating at a large population size, 500 randomly selected individuals were used to initiate four separate population cages, such that there were two replicated predation selection treatments and two no selection treatments. All

four populations were treated identically (other than predation exposure), maintained within 200 mL bottles with a molasses-cornmeal-yeast media at 24°C and 60% humidity, on a 12 hour light (photophase), 12 hour dark (scotophase) cycle. A subset of the initial population was stored in ethanol until sequencing (2.2.3) for reference to the ancestral population.

Selection occurred within the two predation treatment populations, using starved mantids to increase predator veracity. Mixed ages flies were used to remove any developmental effects on survival, with flies randomly sorted by CO_2 anesthesia and allowed 24 hours to recover before any predation bouts. 25 recovered flies were added to a predation cup with 5 mantids via a funnel and left for 24 hours at 18.5°C, 60% humidity, starting during the light, and experiencing 12 hours of darkness and 12 hours (split time) of light. Predicted mortality for each generation was approximately 40% (an observed range of 10%-80%). To limit drift, enough predation cups were set up to ensure a large number of survivors to use for egg laying (150–400 individuals). Controls were treated the same, but without predators present within the cups. Population sizes were matched between treatment and control replicates, such that there was a matching number of males and females to the corresponding selection replicate for egg laying (i.e replicate 1 for both treatments has similar population sizes when egg laying). All surviving individuals from the predation treatment, and the randomly counted controls were added to separate 30 cm^3 BugDorms-43030, and allowed to recover for half an hour. The populations were then allowed to lay a sufficient number of eggs upon fresh food bottles, after which only the bottles were removed from the cages and stored at 24°C and 60% humidity, where the larva could develop for the next generation

to continue this process. This was repeated each generation, with a subset from certain generations stored within ethanol for DNA extraction (2.2.3).

2.2.3 Sequencing

DNA extraction of populations occurred at MSU by Dr. Michael DeNieu and Mauricio Losilla using the Zymo DNA extractor for insects. Three independent extractions were done for each population and generation that had been stored on ethanol previously, with 20 flies used per extraction (10 males, 10 females) for a total pool size of 60 flies. Samples were submitted to RTSF (Research Technology Support Facility) Genomics Core (MSU), where next-generation sequencing libraries were prepared (Illumina TruSeq Nano DNA Library prep kit) and samples were loaded onto two lanes of an Illumina HiSeq 2500 rapid flow cell (version 1). Sequencing was done with Rapid SBS reagents in a 2x150bp paired end format, with the bases called using Illumina Real Time Analysis RTA v1.18.61, and converted to FastQ format. Groups of samples were sent in for sequencing in 2012 and 2015 for three generational time points, generation 38, generation 77 and generation 115, for 12 total sequences. The base population was also sequenced multiple times for greater coverage to uncover the most initial variation that was present in the starting population, for 13 total populations used in analysis (2.2.4).

2.2.4 Data Cleaning

All subsequent analysis was completed either on the Golding server at McMaster University (ssh info.mcmaster.ca) or on personal computers within the laboratory, following a similar workflow to Popoolation2 (Kofler *et al.* 2011a). On the many FastQ files generated by Illumina sequencing (2.2.3), initial quality control checks (md5sum and FastQC) were completed, followed by data cleaning and set up for future analysis. The first step was removing low quality reads and adapters from the FastQ files using Trimmomatic (v 0.33; Bolger *et al.* 2014), going through each populations forward and reverse reads. A minimum length was imposed (36) and an adaptive quality score was used to balance quality with length, set at an intermediate between strict and stringent (0.5). Following this, all the reads from the populations were mapped back onto a reference genome to piece back together, using *Drosophila* version r5.57 (from flybase.org) as the reference genome.

When mapping back to a reference sequence, there is a suite of available software with different mapping algorithms, and specific parameters for utilization. Kofler *et al.* (2016a) found that different mapping algorithms identified varying false positives when compared to each other. The proposed solution to find regions that are true SNPs is to intersect the results from multiple mapping algorithms and find the shared SNPs. Kofler *et al.* (2016a) found the best performing mappers were bwa mem (Li and Durbin 2009), novoalign (Novocraft 2014) and clc4 (CLC bio 2015), but also noted bowtie2 (Langmead and Salzberg 2012) was generally accurate in combination with other mappers. As I began the process of mapping before this information was available with bwa mem, the mapped reads using bwa mem is one mapper used as a comparison. Bowtie2 was readily available on the

Golding server and as it was found to be a good comparison, it was selected as the second mapping algorithm (Kofler *et al.* 2016a). Novoalign was generally the best performing mapper throughout the study by Kofler *et al.* (2016a) and was used as an alternative mapping algorithm, but due to logistical reasons, could not be completed in a reasonable time period.

The Burrows-Wheeler Aligner using the MEM algorithm (bwa mem) uses Burrows-Wheeler Transform (BWT) for matching reads, is similar in accuracy to Novoalign, and in speed to fast mappers, such as Bowtie2 (Kofler *et al.* 2016a, Langmead and Salzberg 2012, Li 2013). Bowtie2 follows a BWT as well and is a quick and memory efficient aligner of sequence reads (Langmead and Salzberg 2012). Bowtie2 and bwa mem run simply by calling the reference genome and the trimmed reads from running Trimmomatic. Throughout the methods from here, the process will be described from step to step for generic SAM, BAM and other files, but all bwa and bowtie2 files are separate and completed independently and do not intersect unless otherwise specified (2.2.6).

The output Sequence Alignment/Map (SAM) files from mapping were converted to Binary Alignment/Map (BAM) files to save memory space and for quicker analysis using samtools (Li *et al.* 2009), while also filtering for a mapping quality score (-q) of 20. As sequencing was completed on two lanes of the Illumina flow cells, the two lanes needed to be merged into one file. SAMtools merge (Li *et al.* 2009) was used to merge lanes together, and this process was also used to merge the different files from the re-sequencing of the ancestral population. When sequencing with Illumina, duplication events can occur, either within PCR (Polymerase Chain Reaction) or optical duplicates due a cluster being identified as two during Illumina

image analysis (Kofler *et al.* 2016b, Gautier *et al.* 2013). In order to mark duplicates for removal, Picard tools was used. First, Picard sorted the BAM files for use with Picard tools MarkDuplicates, which removed any marked duplicated regions. Finally, one last quality check was used to ensure the final 13 sequence files (one per population) were accurate, removing any unmapped reads and creating 13 .bam files.

Up until now, the pipeline has closely followed that of Popoolation2 (Kofler *et al.* 2011a). Now I strayed from these methods and implemented the Genome Analysis Tool Kit (GATK) Indel Realigner (McKenna *et al.* 2010) to reduce the mismatches that may have occured when mapping, specifically which may cause many SNPs to be called around a misaligned insertion or deletion (Indel). This process first targets regions that may need realignment, followed by a realignment of the sequence within those targeted regions (DePristo *et al.* 2011, Van der Auwera *et al.* 2013). This leaves 13 final .bam files that have high quality and alignment and can be used for variant calling and finding regions under selection.

2.2.5 Population Sequence Analysis

I measured the variation of the ancestral base population, which was calculated using Popoolation1 (Kofler *et al.* 2011b). After converting the final BAM file into a mpileup (SAMtools mpileup function, Li *et al.* 2009), using a sliding window approach (window size 10000), Tajima's π was calculated within each window, indicating the nucleotide diversity within each 10000bp block. The same approach was attempted for generation 115, but only with bwa mem mapping.

For comparisons of populations, all 13 sequence files (for one mapper) were combined into one mpileup file using SAMtools mpileup (Li *et al.* 2009), which puts all sequences together into one file containing necessary information (chromosome, position reference base, coverage etc.) along with the variation (in comparision to the reference sequence) for each sample (match, mismatch, indel). As the ancestral state of these populations is available (base population with high coverage), I am able to examine the more interesting analysis on variant selection that has evolved over years from the initial allelic variation. For analysis, this mpileup file is converted to a synchronized file using Popoolation2 scripts (Kofler *et al.* 2011a) which outputs a file showing the position, chromosome, reference base and the allele counts for each of the 13 populations in the format A:T:C:G:N:del. This .sync file could be used for analysis with Popoolation2 scripts but instead I chose to write custom R scripts for analysis to run with R versions 3.3.2 (local) and 3.2.2 (Golding server) (R Core Team 2016).

For custom analysis, I first filtered the two synchronized files (one for bwa mem, one for Bowtie2), which included removing regions that were not a priority nor of interest. Any heterochromatic regions and mitochondrial regions were removed from the files and left out of in further analysis, as I was interested mostly in the 6 major chromosomal regions of *Drosophila*; 2L, 2R, 3L, 3R, 4, and X. To work with these regions of interest, these large files needed to be split to create files of manageable sizes. This was mostly due to the temporary memory capacity of the Golding server, which could not work with this data set in R. The file was broken up into separate chromosomes, and these were split into 10–11 approximately equal size files.

These smaller .sync files were read into R and cleaned to output a .csv file for use later. Firstly, for the planned generalized linear model, the ancestor was copied 3 times, such that each population had an associated generation 0 to the treatment and replicate (although all base replicates were the same). I then identified the major and minor allele counts based on the ancestor, such that each population has a count that corresponds to the ancestral major allele, and a minor allele count that corresponds to the ancestral minor allele, meaning a generation can have a minor allele with a count greater than the major count. This process also identified the base of the major/minor allele for each generation/population. I wanted to ensure that the minor allele count for each population was high enough to decrease the likelihood of a mistaken count. This was filtered by ensuring any position kept had a minor count of at least 5 across all populations/generations, using 5 as a chosen limit to reflect the possibility of a small counts being present in the ancestor and maintained in some populations after many generations.

These filtered files were then run through a loop that ran a generalized linear model (glm) for each position:

```
1 glm(cbind(Major_count, Minor_count) ~ Treatment*Generation)
```

The data at each position can be thought of as a binomial response (major or minor allele count) for different treatments and replicates at different time points (generations). For initial analysis, replicate effects were disregarded, as this allows for a precursory scan of the genome for regions found to have a significant change seen in both replicates. These positions are possibly selected upon as important regions to survival, experiencing convergent selection between the two predation selection replicates. The coefficients for each position (Estimate, Standard Error,

z-value and p-value) was saved into a data frame for the Intercept, Treatment, Generation and Treatment:Generation interaction, and this data frame was written into another .csv file.

2.2.6 Combining Both Mappers Data into One File

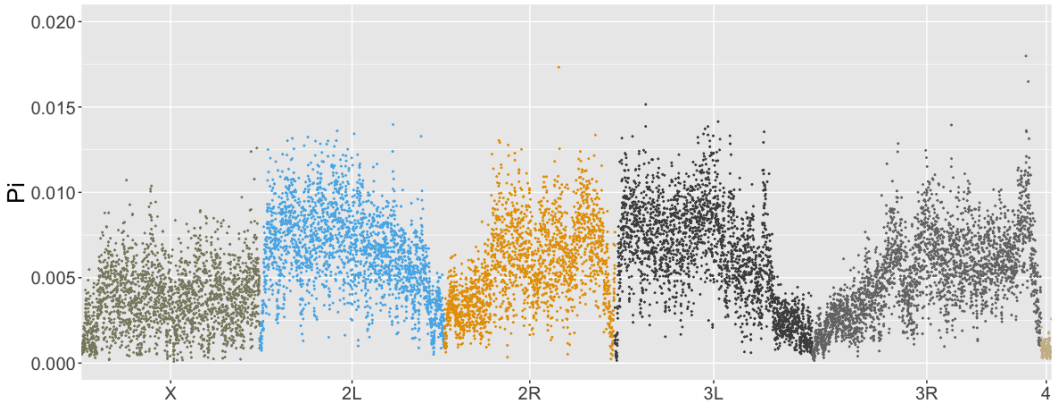
Up until now, all processes were completed twice, once for bwa mem files and once for Bowtie2 generated sequence files. When comparing the two mappers, the method by Kofler *et al.* (2016a) proposed the use of less significant p-value between the two for their Fisher's exact test to remove outliers from the data. This is expected to not only reduce the number of false positives called across the genome, but I expect may reduce any real sites called as this may conservatively remove real sites of significant change. I present results that take the average between the two mapping algorithms, as this may be a more viable method, where there is less stringency between the two mappers and potentially keeping most real SNPs under selection due to predation.

2.3 Results

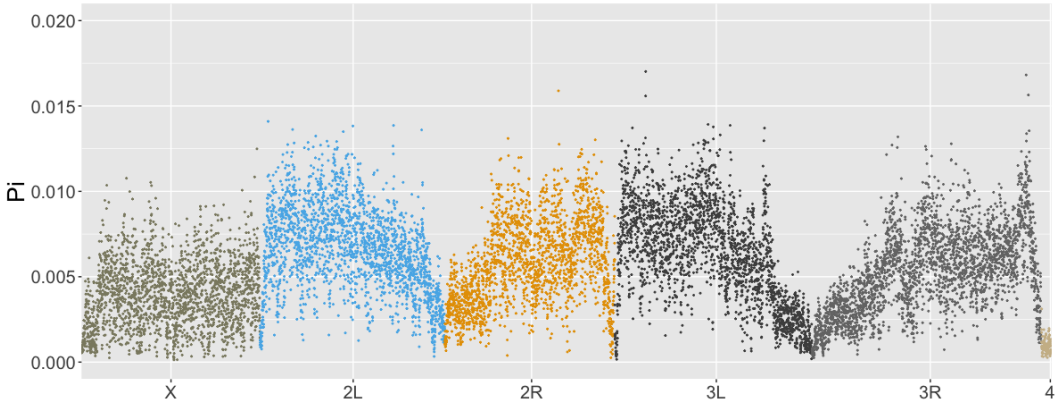
2.3.1 Ancestral Variation

For the ancestral base population, Tajima's π was calculated for both mappers separately as an observed measure of initial diversity across the genome (Fig. 2.1). To highlight differences present between mapping algorithms, a plot with a loess

spline for the two mappers for average diversity is shown (Fig. 2.2). These plots can highlight many key aspects of the ancestral population, including more genetic diversity in autosomes (2L, 2R, 3L and 3R) compared to the X chromosome, and the small 4^{th} chromosome. The drops between 2L/2R and 3L/3R are at the centromere for that chromosome (2^{nd} and 3^{rd}), as well as the opposite ends of these two chromosomes showing a drop in diversity as well (at the telomeres).



(A)



(B)

FIGURE 2.1: Ancestral variation present across the genome (X, 2L, 2R, 3L, 3R and 4th chromosome), measured as Tajima's π, a measure of nucleotide diversity for a non-overlapping window of 10000 base pairs for (A) bwa mem mapping and (B) bowtie mapping.

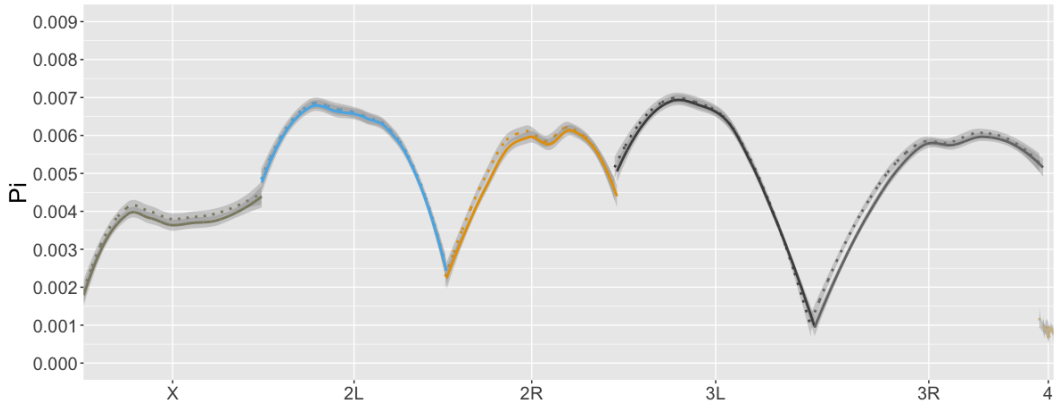


FIGURE 2.2: Overlay plot of both mapping software using a loess spline (ggplot2) for π between the two mappers. Dotted line represents Bowtie2, and solid line represents bwa mem.

2.3.2 Evolved Response in due to Treatment Effects due to Predation

The analysis of the nucleotide diversity (Tajima's π) was attempted with the .bam files from bwa mem mapping at generation 115, however this analysis did not appear to work and will be attempted in future analysis. The hope was to express the diversity in the final generation sequenced for each population, but unfortunately this could not be shown. It appears the π value calculated for each window size was "*na*" within the output files from the Variance-sliding.pl script used from Popoolation1 (Kofler *et al.* 2011b), which I am unsure whether this is due to an error in the set up when running each file, or due to reduced nucleotide diversity in the 115^{th} generation that will have each 10000 bp window show no diversity (output a "*na*").

For each generational time point and selection regime, the output from the glm was saved and used to calculate the average p-value (between bwa mem and

Bowtie2 mappers) for sites of significant change due to predation selection vs. the controls (Fig. 2.3). Note a region at the end of the 3R chromosome was not included, as the large file did not complete the glm model. The positions along each chromosome is shown with the associated -log10 of the mean p-value. Significant peaks can be seen across the genome, with many peaks being located far from the centromere and are generally clustered with other regions.

Although a false discovery rate to find a significance threshold was planned, the large data set was unable to complete this process in an effective amount of time. Instead, the top 1% significant positions of each chromosome is shown in dark grey, and the top 0.01% significant positions of the genome is highlighted in green (Figures 2.4, 2.5, 2.6, 2.7, 2.8 and 2.9). These values are selected to aid in the visualization of each chromosome differentiation and possible selected sites within these chromosomes due to predation selection

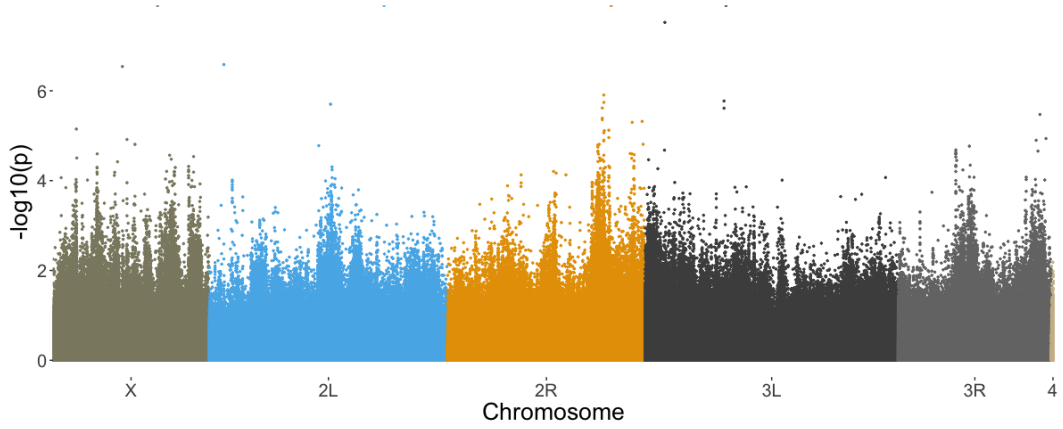


FIGURE 2.3: The genome of *Drosophila melanogaster* with -log10(p-values) of evolved population of flies showing positions of significant variation caused by different allele frequencies between predator selection treatments or the associated control treatments with no selection.

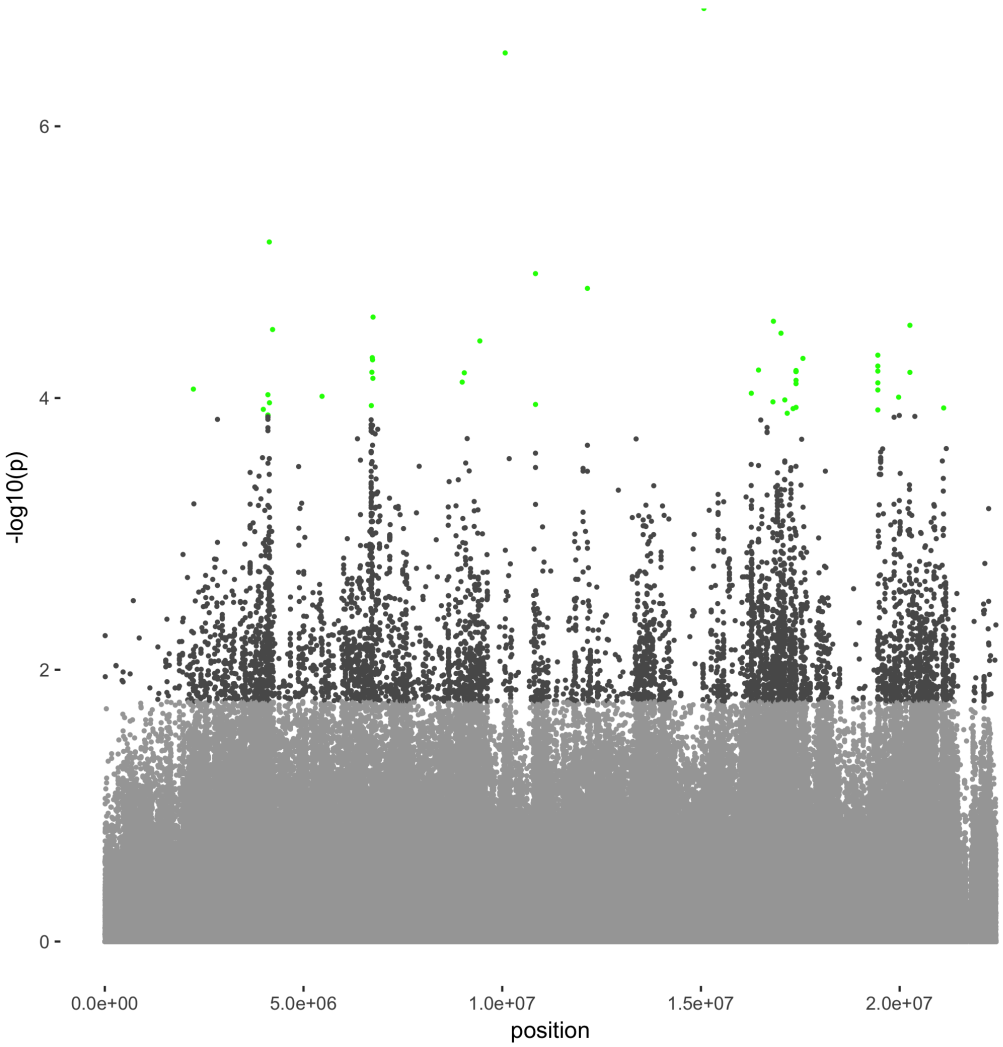


FIGURE 2.4: **X chromosome**; -log10(p-values) for positions along the X chromosome for *Drosophila* evolved populations, showing the bottom 99% positions in terms of significance (light grey), the top 1% significant regions (dark grey) and the top 0.01% significant regions (green).

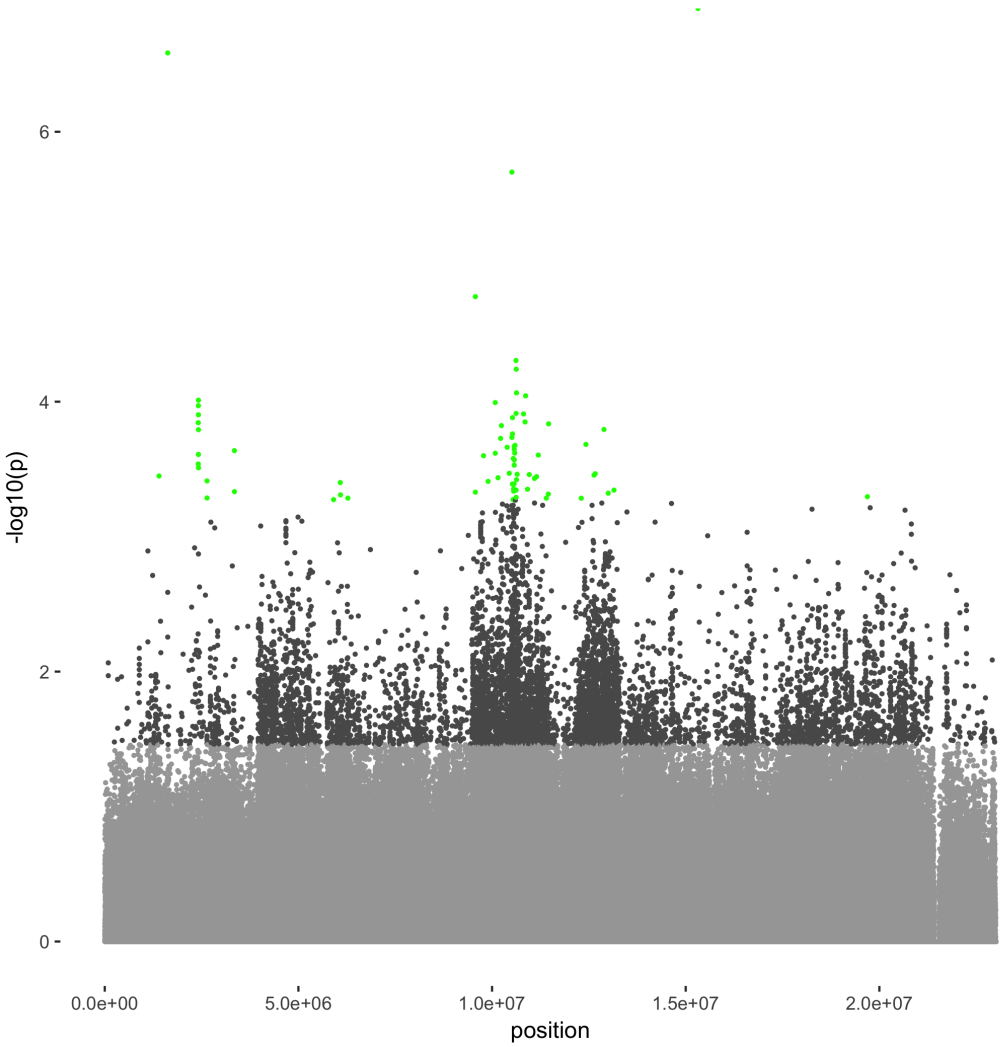


FIGURE 2.5: **2L chromosome**; -log10(p-values) for positions along the 2L chromosome for *Drosophila* evolved populations, showing the bottom 99% positions in terms of significance (light grey), the top 1% significant regions (dark grey) and the top 0.01% significant regions (green).

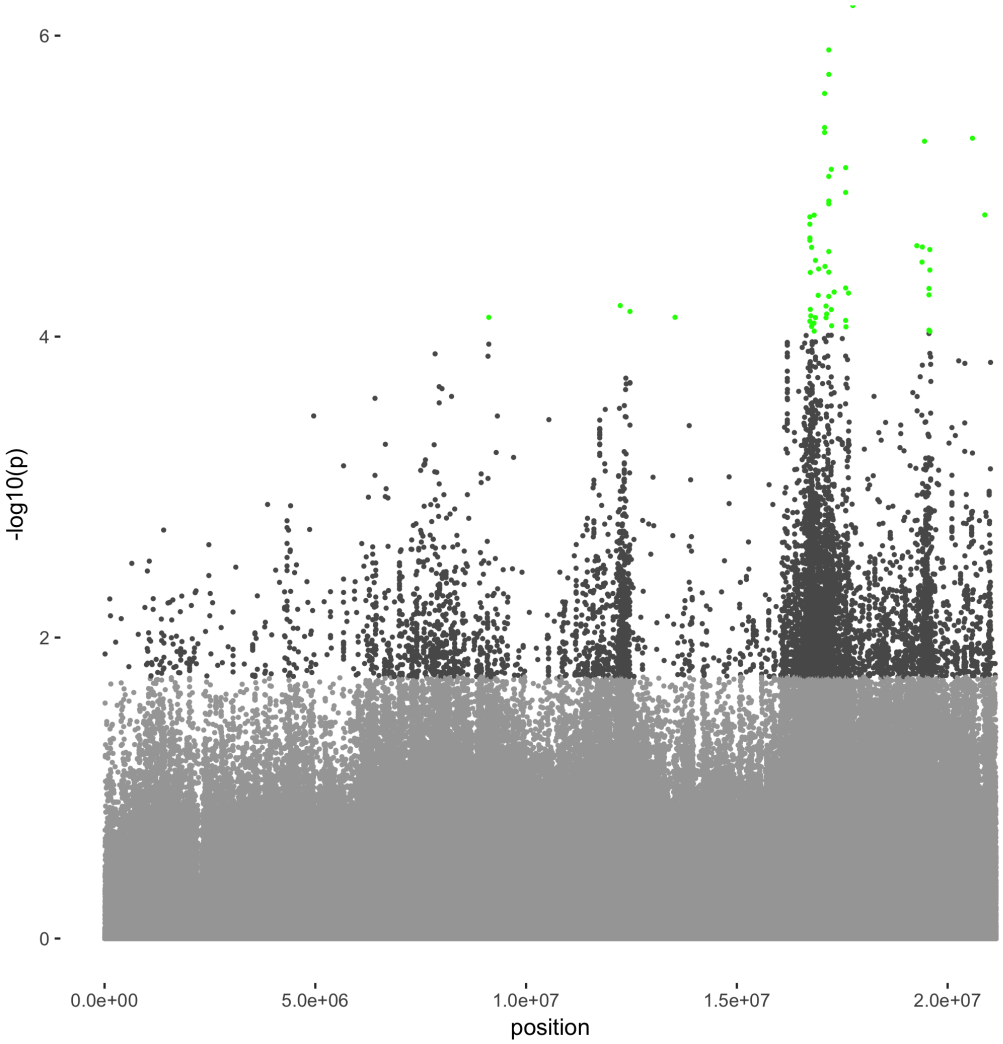


FIGURE 2.6: **2R chromosome**; -log10(p-values) for positions along the 2R chromosome for *Drosophila* evolved populations, showing the bottom 99% positions in terms of significance (light grey), the top 1% significant regions (dark grey) and the top 0.01% significant regions (green).

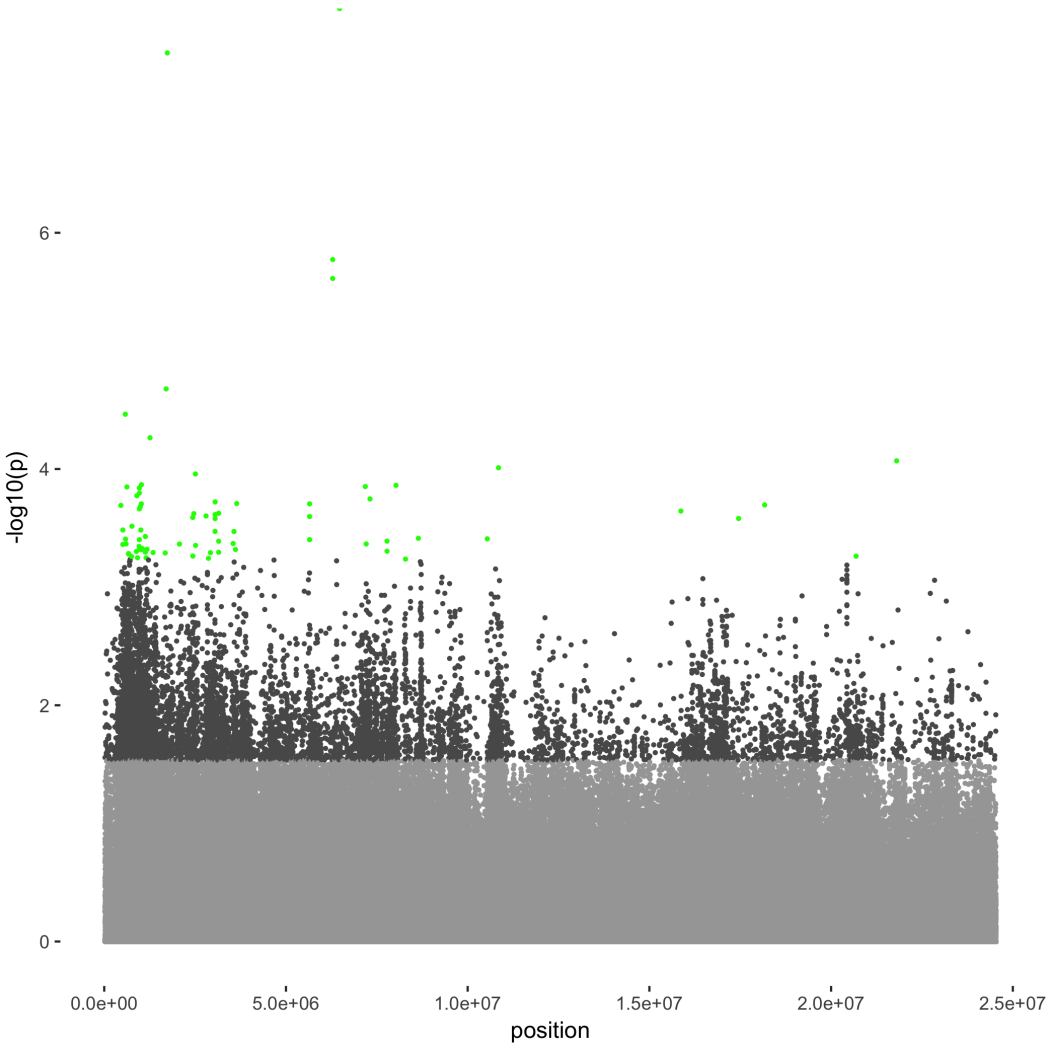


FIGURE 2.7: **3L chromosome**; -log10(p-values) for positions along the 3L chromosome for *Drosophila* evolved populations, showing the bottom 99% positions in terms of significance (light grey), the top 1% significant regions (dark grey) and the top 0.01% significant regions (green).

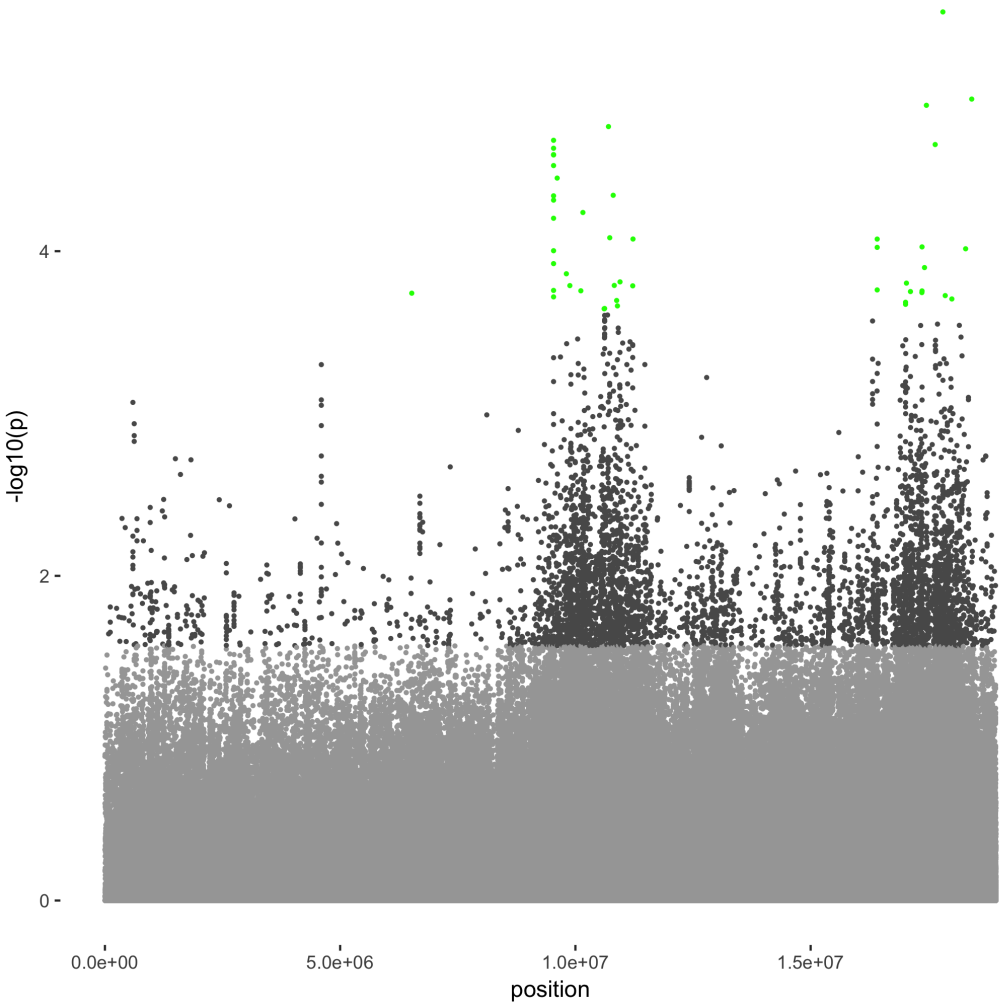


FIGURE 2.8: **3R chromosome**;-log10(p-values) for positions along the 3R chromosome for *Drosophila* evolved populations, showing the bottom 99% positions in terms of significance (light grey), the top 1% significant regions (dark grey) and the top 0.01% significant regions (green). This section of the chromosome is incomplete, and due to logistical reasons, a section at the right end could not be included in this plot.

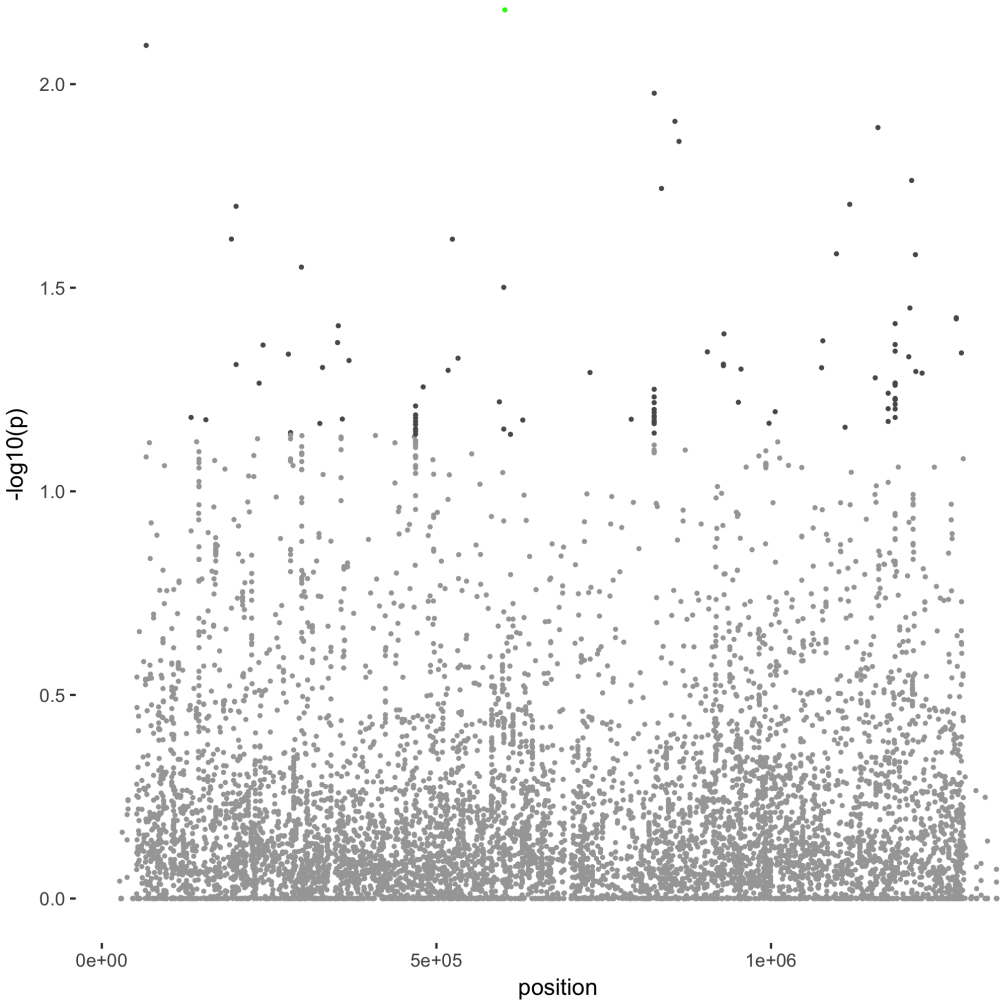


FIGURE 2.9: 4^{th} **chromosome**; -log10(p-values) for positions along the 4^{th} chromosome for *Drosophila* evolved populations, showing the bottom 99% positions in terms of significance (light grey), the top 1% significant regions (dark grey) and the top 0.01% significant regions (green, only one position as the chromosome is quite small).

2.4 Discussion

Looking at figure 2.1, it is apparent that there is variation across the ancestral base population. This genomic variation is predicted to contain positions that will be selected upon to increase or decrease in frequencies across the population based on the beneficial or detrimental role they play for predation survival. The outline of the genome looks as expected, with less variation seen on the 2^{nd} and 3^{rd} chromosomes near the telomeres (drops at left edge of 2L and 3L, as well as right side of 2R and 3R) and the centromeres (drops between 2L/2R and 3L/3R). As well, little variation is seen on the 4^{th} chromosome (the smallest on the far right), and relatively more variation on the autosomes is seen compared to the X chromosome (Casillas and Barbadilla 2017, Nolte *et al.* 2013, Orozco-terWengel *et al.* 2012).

The comparison of the variation that was able to be detected between the two different mapping methods appears to be similar (Fig. 2.2), but with close inspection, some variation on the fitted line can be seen. While the overall pattern of genomic variation is consistent between the two mappers, there is an increase in the average nucleotide diversity over the chromosomes for Bowtie2 mapping, seen with the dotted line lying above much of the solid line (bwa mem). This overlay between mapping with bwa mem and Bowtie2 highlights the importance for multiple mapping, as the measures of diversity is completed in 10000 base pair windows, likely meaning large differences are causing the shifting of splines in figure 2.2. Therefore, although there appears to be similar results between mappers, I do not remove either from analysis, as significant differences at single positions

may be called as false positives, causing the non-overlap between lines. As the ancestral population was sequenced at multiple times for a high and accurate coverage to discover rare variants, the similarities between the two mappers may yield the more potentially real positions of significant variation. When averaging the p-values between the bwa mem and Bowtie2, the more similarity in accuracy between the two will allow for more real variants not being filtered out of the sequences and to also keep false positives out of the analysis.

The genome of *Drosophia* populations appears to be under selection, with many positions showing significant differences between predator selection treatments, and no predator selection treatments (Fig. 2.3). The significant variation between treatments is showing overall significant differences to each single nucleotide polymorphism (SNP) due to mantid selection. The results do not indicate the direction the genome is evolving, but rather the overall differences predation selection causes compared to controls. This variation shows many peaks of greater significance, possibly regions that are strongly selected upon by mantids for either increases (or decreases) in beneficial traits expressed within these regions.

The positions found to have significant differentiation between population treatments is widespread, with highly significant variable positions seen on almost every chromosome, with the exception of the 4^{th} chromosome (Fig. 2.9). This chromosome has mostly lower significant values, with the largest -log10 p values less than 2, when most top values across the other chromosomes are above 2 (top 1% in dark grey in figures 2.4 – 2.8). The 4^{th} chromosome is the smallest chromosome in *Drosohila*, has a low recombination rate and contains only a few genes, which may explain the relative low variation between treatments seemingly present on

this chromosome (Berry *et al.* 1991). Generally, the 4^{th} chromosome is rarely discussed in genomic studies on *Drosophila* evolution (Fabian *et al.* 2012, Nolte *et al.* 2013, Orozco-terWengel *et al.* 2012, Turner *et al.* 2011).

Significant peaks are seen across the other 5 chromosomes (X, 2L, 2R, 3L and 3R), with the top 1% of significantly different SNPs between predation and control populations shown in dark grey on each chromosome (Fig. 2.4 – 2.8). Looking at the top 0.01% significant SNPs on these same plots (highlighted green), these most significant positions generally lie atop the clustered peaks in dark grey.

Looking at the associated chromosome arms of 2L and 2R (Fig. 2.5 and Fig. 2.6) as well as 3L and 3R (Fig. 2.7 and Fig. 2.8), it appears the positions with the greatest change between treatments are found at the regions further from the centromere (far left in Fig. 2.5 and Fig. 2.7, far right in Fig. 2.6 and Fig. 2.8). This may be the product of the initial base population showing lower genetic diversity within close to centromeric regions (Fig. 2.1) (generally 2-4 fold less diversity, Casillas and Barbadilla (2017)). As there was lower standing variation initially in the population to be selected upon by predation over the years, and such, a less significant deviation between treatments could be observed. It may be that regions close to the centromere did not initially house many potentially beneficial alleles to predation threat in the ancestor population that selection could act upon. Some E&R studies, like I have found, show the centromeric regions do not vary between laboratory selection regimes (Fabian *et al.* 2012). Others have found regions near centromeres contain many highly significant regions of selection (Orozco-terWengel *et al.* 2012, Turner *et al.* 2011). As the recombination rate at the centromere is lower in *Drosophila* (Comeron *et al.* 2012), any beneficial

alleles selected upon would show high levels of hitchhiking, where large regions of significant differentiation may be seen near the centromere (ex. Orozco-terWengel *et al.* 2012). It is likely that these centromeric locations contained few beneficial allelic variants for predatory survival within the base population that selection could act upon to increase in frequency.

Further analysis into the differentiated locations found with precision to the exact positions showing significant allele frequency changes can be done. This will allow for insight on the genes that may be under selection. Although speculation into direct genes may be early in the analysis, I wanted to examine the largest cluster of significant SNP's observed in figure 2.3, the region of the 2R chromosome, found to be approximately spanning a region of positions 16731302–17751089. Many genes are found within this cluster, including the large gene muscleblind (*mbl*), located at 2R:17,216,549–17,379,376, close to the center of the cluster of highly significant positions. Of the 61 positions in the top 0.01% on the 2R chromosome, 35% of the 61 positions fall in the range of *mbl*. The *mbl* gene has been found to be involved with muscle and eye development (Begemann *et al.* 1997) and female receptivity to mating (Juni and Yamamoto 2009). These processes may be important factors selected within the population. Expression of a gene that allows for more well developed muscles (flight initiation and escape) and eyes (detect predators) may allow for an increase in survivability. As well, females modulating receptivity when encountering a male may reduce any risk associated with mating behaviours. These factors may have lead to the selection populations expressing significant variation in this region.

Looking at direct genes and the associated function may be early, with *mbl*

discussed due to the range of positions that housed this gene matching a large number of positions with large significance. As this gene is quite large, this may also lead to an over representation of significant positions in this gene. However, this highlights the ability that may come about in further analysis, looking to correlate highly significant positions for change within the selection lineages to specific genetic functions. Depending on the possible genes identified, follow up experiments could look into gene knockdown mutant lines and compare the survivability of these lines when exposed to predators (Duffy 2002). As well, other selection regimes with predators (some within the lab) may also be selected for the putative genes, and can be sequenced and compared to see if these genes are under selection as well.

What I have found is evidence mantid predation is altering the genomic structure of these selected populations of flies (Fig. 2.3), selecting on certain variants that are significantly different alleles between predation or control selection treatments. The high risk environment appears to play a role in altering the behaviours in order to not only survive, but still be in a fit enough state to pass on these beneficial traits to future generations.The exposure to predators for fly populations is very long, with up to 80% mortality. Surviving these exposures can be risky, not only to immediate survival, but if they are not leaving the high risk encounters with an overall high fitness, there can be serious costs to flies. The populations are not given a long opportunity after predator exposure to mate and lay eggs for the next generation, so those with the highest fitness after exposure will be at an advantage. Thus, evolution will favour traits beneficial to both survival at an overall fitness most beneficial to both survival of a bout of predation, and future

fitness after the predators are removed.

Obviously this work is ongoing and requires additional research and data analysis to be sure of the effects predator selection is having on *Drosophila*, but it looks promising to find regions of the genome that are being selected within these populations, and possibly identify genes contributing to increased survival.

Chapter 3

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3.1 Chapter 1 Bibliography

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Appendix A

Supplementary Figures of some Experimental Design

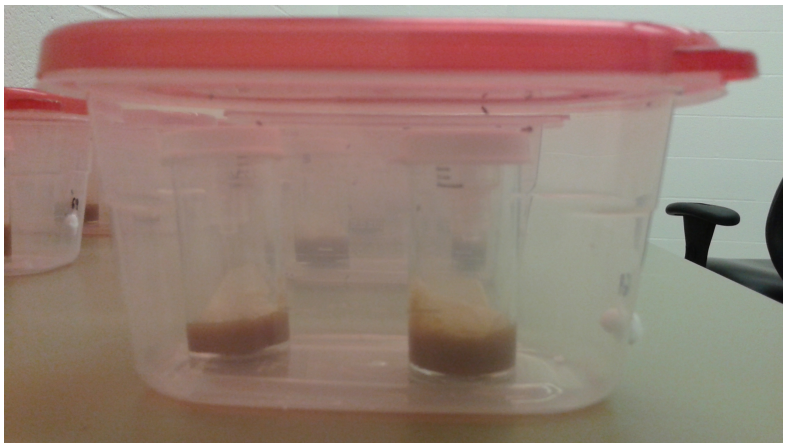


FIGURE A1.1: The 2 vials and *Snaptite* chamber 10 same sex flies were put into and allowed to enter (but not exit due to a funnel lid) a vial with either a spider and food, or only a food vial

FIGURE A1.2: The *Snaptite* chambers in the controlled room with little disturbances, with controlled light, air, temperature and humidity

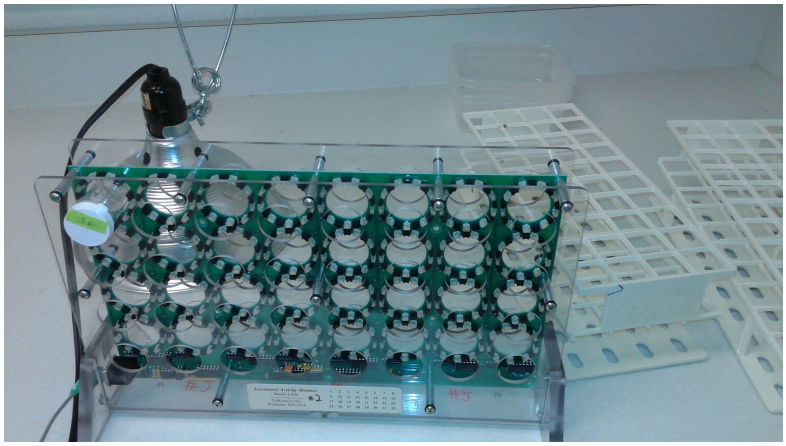


FIGURE A1.3: The **DAM** 32 wells, used to measure activity counts, which is the number of crossings at the center of well. Vials housed like example vial in top left corner, which can hold both the "cue" individuals (predators, crickets etc.) as well as the "focal" individual (*Drosophila* or the predators.

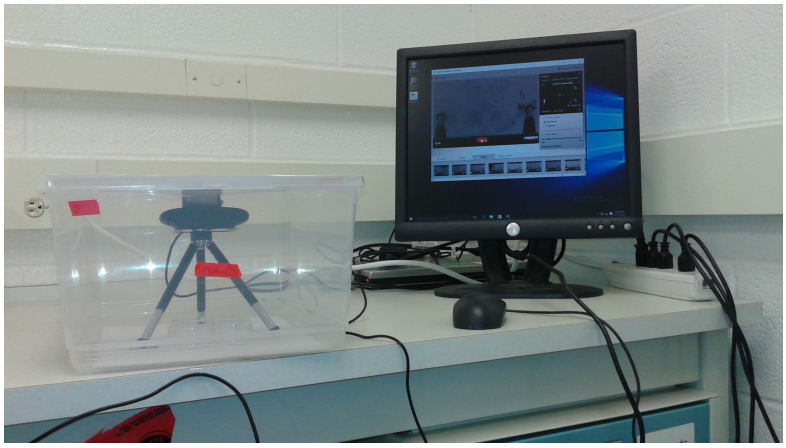


FIGURE A1.4: The set up for mating experiments to record courtship and copulations in the recently caught population of flies. The flies are placed within modified petri dishes within the bin (left) with video recording from above and saved on the computer

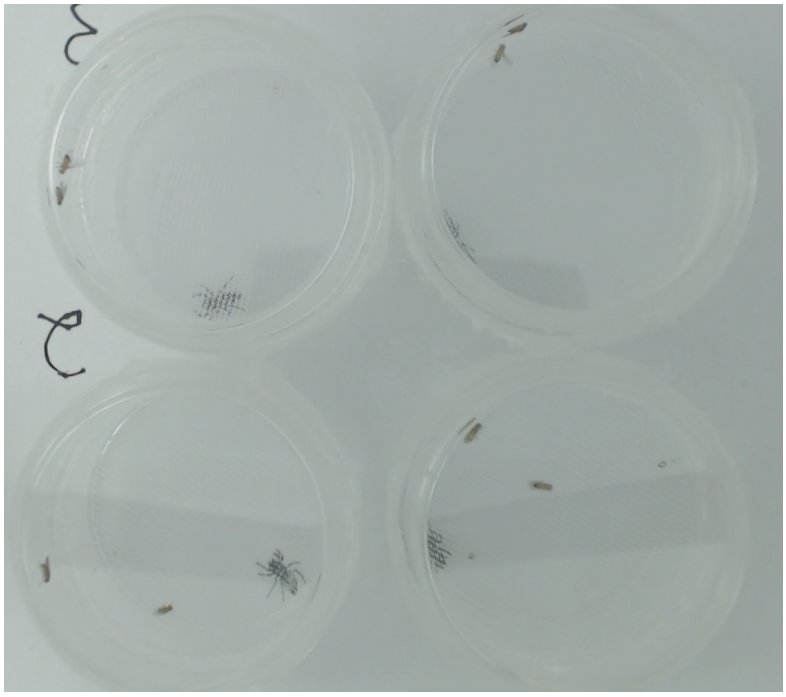
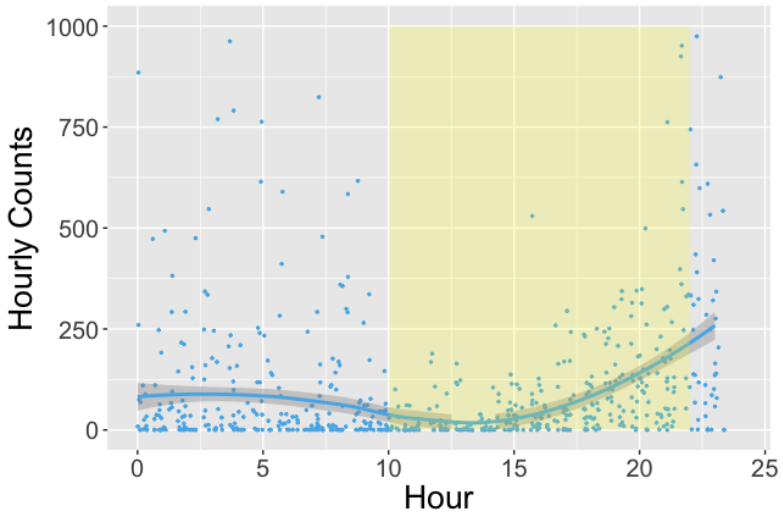
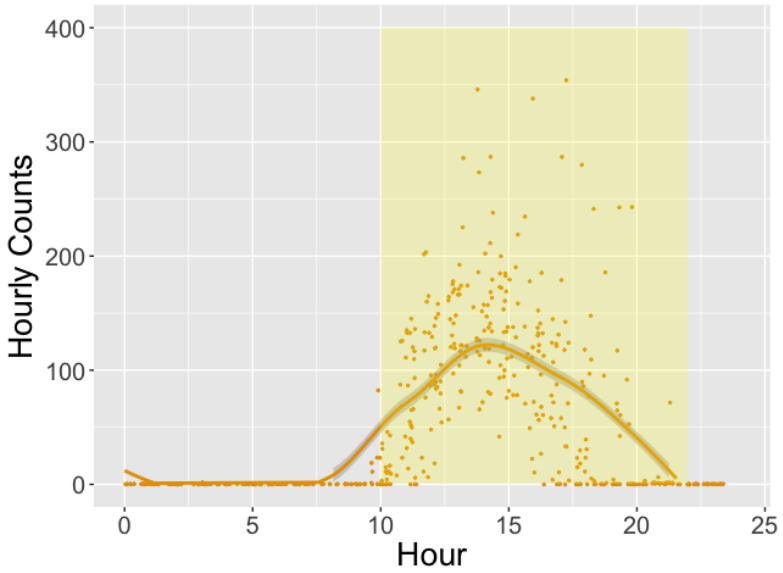


FIGURE A1.5: In groups of four, the courtship and copulation times for a mating pair with either spiders present (as seen here) or no spiders present. The spider is separated from the male and female by thin mesh that should pass olfacotry and visual cues to the mating pair.



(A)



(B)

FIGURE A1.6: Predator hourly activity for both mantids (A) and spiders (B) over 24 hours, with daytime (lights on) represented by the yellow area. A loess ("ggplot2") spline is added for average activity though the day with mantids showing greater activity at night vs. the spiders increase in activity during the day. Shaded area represents the 95% confidence interval