USING EXPERIMENTAL EVOLUTION IN *DROSOPHILA MELANOGASTER* TO TEST PREDICTIONS ABOUT THE ADAPTATION OF PREY TO A NOVEL PREDATOR

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ABSTRACT

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One of the primary questions in organismal biology is how evolution has acted to shape the species that we see in nature. Beginning to address this incredibly complex question requires a diverse set of approaches that can be difficult to accomplish in the wild, in part because it requires a relatively robust knowledge of the evolutionary history of given populations. Though it cannot tell us how existing species have evolved, experimental evolution is a powerful tool because it allows one to track phenotypic and genotypic changes in populations over time in response to a controlled selection pressure. By imposing a particular selection pressure on populations with a known origin, I can test hypotheses about organismal evolution generated by studying patterns in nature. Here I will discuss a series of experiments conducted on populations of *Drosophila melanogaster* that have been evolved under predatory selection by nymphs of the Chinese mantis (*Tenodera aridifolia sinensis*). I first investigated the ability to use phenotypic selection analysis to determine long term evolutionary outcomes. To do this, I measured selection acting on wing size and shape in the base population and then again in the evolved populations after 30 generations of selection, and used this to determine other important morphological and behavioral traits that have likely been targets of selection. I show that evolutionary trajectories are largely predictable, but that unmeasured traits can have profound effects on evolutionary outcomes. I also test the predictions of the risk allocation hypothesis as it pertains to courtship, aggression, and anti-predator behavior. Unlike many previous studies that have focused on learned responses to predation risk, I tested whether populations evolved under differences in variation in predation risk would evolve behavioral patterns consistent with the risk allocation hypothesis. I found that while the hypothesis captured several important aspects of the evolutionary response, the specific predictions failed to accurately describe the actual outcome. my results suggested that the riskiness of different behavioral types played a large role

in determining whether they conformed to the predictions of risk allocation. In my final chapter, I investigate a unique behavior in which flies evolved in the presence of predators reduce their propensity to initiate flight. Though my results cannot conclusively determine the cause of the evolution of this new escape strategy, they do suggest that associations with allometric scaling relationships are important in determining the fitness of the divergent strategies observed in the predator-evolved populations.

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CHAPTER 1

INTRODUCTION

1.1 Premise

One of the primary goals of evolutionary biology is to understand how the organisms that we see in nature have evolved. Classically, researchers have studied patterns of selection, divergence, behavior, morphology, and life history in natural systems in order to generate explanatory hypotheses and develop mathematical theory that would allow the prediction of future change. The comparative method is one of the primary tools for this purpose because it allows one to use existing variation among species or populations to infer their evolutionary history and the processes that might have shaped it. In other words, the comparative method evaluates the evidence left over from past evolution that has already taken place. Though this is a powerful approach that is still widely used today, it is difficult to directly test the hypotheses generated from it (Magalhães and Matos, 2012). Increasingly, experimental evolution is being used so that tests of the predictions of evolutionary theory can be performed (Kawecki et al., 2012). Though this is only one of the many uses for experimental evolution, it is one of the most powerful.

Experimental evolution, also referred to as laboratory natural selection, is one of two major types of laboratory evolution (Fuller et al., 2005). It can be distinguished from the other, artificial selection, because the experimenter presents a population with a specific selection pressure but does not explicitly control the traits that are selected. The experimenter controls the environment in which the populations evolve, and the populations are free to move along any trajectory where there is genetic variation and that is favored by selection. In this way, the evolving populations are able to provide information about the processes that are shaping their evolution. There is no guarantee that replicate populations will evolve the same phenotypes (Lenski and Travisano, 1994). Even if replicates do evolve similar phenotypes, the underlying genetics may themselves be different.

Furthermore, there are legitimate questions about how applicable the artificial environment of the laboratory is to natural systems, and how generalizable the results are beyond the base population used to generate experimental treatments (Harshman and Hoffmann, 2000).

However, with sufficient replication and precise control over the selective environment, experimental evolution is one of the few methods that allows the experimenter to measure direct and indirect responses to specific selection pressures on populations with a known history over long time periods. It also allows for the identification of correlated responses to selection that can provide insights about pleiotropy and interactions among traits that may not have been expected to be related (Fuller et al., 2005; Harshman and Hoffmann, 2000). This can help to reveal the mechanisms that underlie the biological processes that are under investigation. Indeed, experimental evolution has been used to successfully investigate life history (Prasad and Joshi, 2003), aging (Stearns et al., 2000), reversibility of evolution (Teotónio and Rose, 2001), and the evolution of novelty (Blount et al., 2008), along with many other phenomena.

In the subsequent sections, I will discuss several studies that have used experimental evolution to test evolutionary theory related to behavioral phenotypes, which are the primary focus of this dissertation. Though predation is the main selection pressure used to evolve the populations described in later chapters, the principal goal was to use this selection pressure to understand questions related to behavioral evolution, not to specifically probe aspects of predation. Therefore, I will focus on studies that have used experimental evolution to test hypotheses about the evolution of sex and the evolution of learning because, even though the selection pressures are different, the approach and methodology are largely interchangeable. These examples will demonstrate the breadth and depth of questions that have been and are currently being pursued by others in the field. I will discuss the insights that have been gained from them and the caveats with which the results should be viewed. I will then address the approach I have taken in using experimental evolution to test hypotheses about the predictability of selection, the effects of variation in predation risk, and the level of organization at which selection acts.

1.2 Evolution of learning

One way to define learning is that it is a change in an organism's behavior based on a past experience (Kawecki, 2010). Unlike other forms of plasticity, learned responses are developed over the course of a the lifetime of an organism. However, the ability to learn still must have evolved. To begin to address questions about the evolution of learning, Mery and Kawecki (2002) subjected an outbred population of *Drosophila melanogaster* to an experimental procedure where flies that performed better at an associative learning task were at a selective advantage. It had been established that artificial selection could be successfully used to increase the ability to learn. They instead asked whether flies exposed to natural selection would also increase their ability to learn.

Adult flies were given the choice between two different substrates on which they could oviposit. One set of media was prepared with orange juice and the other was prepared with pineapple juice. During an initial conditioning phase, quinine was added to one of the substrates. Quinine has a bitter taste that the flies avoided but could not smell because it was not volatile. In a second phase, the flies were again presented with both substrates, this time neither contained quinine, in order to provide a buffer between the conditioning and testing phases. During the third and final testing phase, flies were presented both substrates lacking quinine and eggs laid on the flavor that did not contain quinine in the conditioning phase were kept and passaged for the next generation. The quinine-containing substrate was alternated each generation so that there was not selection on innate preference for a particular flavor.

After approximately 50 generations of evolution, the experimental flies evolved a higher rate of learning as measured by the proportion of eggs laid on the correct media, and the effect of conditioning decayed more slowly than controls. This experiment showed that under conditions expected to lead to the evolution of increased learning ability, flies did, in fact, evolve this behavior. Though this may seem like a trivial result—nearly all metazoans show some capacity to learn and undoubtedly selection has played a role in this—experiments like this one provide a link between a particular selection regime, and an evolved response. In addition, unlike many other experiments, the evolved populations can form the basis for additional experimentation.

1.2.1 Experimental evolution populations are a resource

A valuable aspect of experimental evolution is that, with the proper controls, it generates populations that differ primarily in their experience of a single selection pressure. As a result, differences between control and experimental populations can reasonably be assumed to be due to the environment created by the researcher. Because of this, Mery and Kawecki (2003) sought to answer the question of whether the increased ability to learn in their populations came at a cost. It was expected that the energy and maintenance costs of the neuronal structures associated with learning ability would reduce fitness in other areas. As they predicted, they found that the high learning populations had reduced larval competitive ability as compared to controls. It has since been discovered that there is also a negative relationship between learning ability and longevity in these populations (Burger et al., 2008).

Experimental populations can also be used to ask questions that would be difficult to address otherwise. There is some evidence that repeated tasks are associated with specific increases in learning and memory, but there is little experimental evidence that selection for learning in one context generalizes to another (Dukas, 2004). The high learning populations provided the ideal situation in which to test this hypothesis because the control populations provide a contrast that seldom exists in natural systems. Mery et al. (2007) tested these flies for their ability to learn in a different context. This time they paired an odorant cue with a violent, mechanical vibration. During the conditioning, an odorant was pumped into the chamber, which was then shaken. The chamber was then cleared with air and a second odorant was added. After being cleared with air, the flies were tested with both odors in a t-maze. They found that the flies that were selected for increased learning also performed better in this test even though the aversive stimulus and behavioral action differed.

1.2.2 Experimental evolution can be replicated

Unlike natural systems, the selection regimes used to create experimentally evolved populations can be replicated. In some cases this is done by the same investigators in order to modify an aspect

of the selection regime or the data collection in order to address a slightly different question. In addition to the populations described in Mery and Kawecki (2002), additional populations were maintained in which there was selection for innate preference (Mery and Kawecki, 2004). Instead of alternating the flavor substrate collected, for each population one substrate was always collected (always orange / always pineapple). In some populations, the flies were given the opportunity to learn. For example, if orange was the substrate that was picked, the quinine would always be in the pineapple during the conditioning phase. For the remaining populations, no quinine was added during the conditioning phase, so the flies had no opportunity to learn the correct choice.

Like many other experimental evolution experiments, the evolved patterns did not support the theoretical predictions. Populations that had the opportunity to learn showed an increase in their ability to do so, despite the fact that predictions based on the costs of learning suggested that they should not have. In addition, even though all populations evolved innate preference for their selected flavor, the ability to learn facilitated the evolution of preference for pineapple but constrained the evolution of preference for orange. However, far from being disappointing, these results point to important aspects of learning that we still do not understand.

In this case, researchers from a different lab replicated the learning environment, but made adjustments to gain a deeper understanding of the nuances in the evolution of learning. The previous work had operated under the framework that variability should select for learning and consistency should select for innate preference. Dunlap and Stephens (2009) suggested that concepts they referred to as the reliability of experience and fixity of the best action explained the potentially counter-intuitive results of Mery and Kawecki (2004). Reliability described the extent to which the conditioning predicted the correct action during testing. The fixity described the extent to which the correct action is the same from generation to generation. Under their formulation, any context with high reliability, such as that used by Mery and Kawecki (2002, 2004), should select for the evolution of learning. It is only when reliability is low, that high fixity will not favor learning, and this is exactly what their data showed. These results underscore the importance of replication in experimental evolution because, to some extent, populations can be re-evolved to address new

questions or to address the same question in a different way.

1.3 Evolution of sex

The past section focused on the depth of insight that can be gained from a single system. This section will focus on some of the breadth of questions that can be addressed. using experimental evolution. One of the most basic questions about the evolution of sex is why organisms have sex at all (Manning, 1984). Because of the twofold cost of males, selfing organisms should out-compete sexual organisms by producing more offspring. Several hypotheses exist for why outcrossing is so prevalent, but support for these hypotheses is often based more on the fact that outcrossing is so prevalent, not because there is a wealth of direct evidence for them. Experimental evolution provides a way to test hypotheses such as these that might be impossible to investigate any other way.

Morran et al. (2009) tested the hypotheses that outcrossing prevents the fixation of deleterious alleles and facilitates adaptation to novel environments. To address these questions, they used *Caenorhabditis elegans* worms that were to be obligately outcrossing, obligately selfing, or capable of both. To test the first hypothesis, these worms were exposed to a mutagen and allowed to evolve, and to test the second hypothesis, worms were exposed to a novel environment. They found that outcrossing lines fixed fewer deleterious alleles than selfing lines and had higher fitness in the new environment, supporting both of these hypotheses. In addition, they found that facultatively outcrossing lines increased the rate of outcrossing during the evolutionary process providing further support. Though these data are unsurprising, the ability to explicitly test fundamental assumptions is extremely important.

1.3.1 Uncoupling forces of selection

Studies on sexual selection are also often focused on conflict. This conflict could be between sexual selection and natural selection, male fitness and female fitness, or multiple males. One of

the most powerful aspects of experimental evolution is the ability to control selection in ways that would be extremely difficult in natural systems, so that these various levels of selection can be uncoupled.

Rundle et al. (2006) investigated the relationship between natural selection and sexual selection in order to better understand the effect of sexual selection on aspects of fitness unrelated to mating. The good genes hypotheses states that sexually selected traits are honest signals of male fitness. If this were operating, sexual selection should reinforce natural selection leading to more rapid adaptation. If sexual and natural selection were antagonistic, the opportunity for sexual selection should slow the rate of adaptation. To test this, they created populations of *Drosophila serrata* in which neither sexual nor natural selection were permitted, only sexual selection was permitted, only natural selection was permitted, and both forms of selection were permitted. They found that sexual selection did not facilitate adaptation. Populations exposed to natural selection adapted faster than those without. However, sexual selection did not hinder adaptation in the population with both.

In a related experiment, Chenoweth et al. (2008) investigated the effects of natural and sexual selection on sexual dimorphism in cuticular hydrocarbons. The classical assumption was that sexual selection led to increases in dimorphism primarily by acting on males. Their results somewhat confirmed this hypothesis, in that sexual selection tended to increase sexual dimorphism and natural selection tended to decrease it. However, they found no evidence for sexual selection on males. They only found evidence for sexual selection on females.

Prasad and Joshi (2003) investigated the factors that might affect the expression of sexual dimorphism. In particular, they focused on intralocus conflict. By using stocks containing specialized chromosomal constructs, they were able to create a breeding design in which only males were exposed to selection. Consistent with previous studies (Rice, 1996), males evolved higher fitness and showed greater expression of male traits. Importantly, they were also able to show that when females possessed the male evolved chromosomes, they caused a symmetrical decrease in fitness.

Together, these results show that antagonism between sexual and natural selection, as well as

antagonism between sexes, are important determinants of evolutionary outcomes. However, it is important not to rely too heavily on the specific patterns from one group of organisms. Simmons and García-González (2008) investigated the effects of monogamy on the evolution of testes size in *Onthophagus taurus*. In these beetles, males are dimorphic, in which one morph grows large horns and uses them to defend a nest. The other morph does not grow horns and attempts to sneak copulations from other males. As a result, sperm competition is strong in these beetles just as it in is *Drosophila*.

Experimental evolution populations were initiated in which the mating system was either polygamous or monogamous. After approximately 20 generations, the testes size of polygamous males had increased along with their competitive ability and those of the monogamous males had decreased. These results were not surprising because the proportion of offspring sired by a male is dependent on the number of sperm he produces. In *Drosophila*, accessory proteins are produced and transmitted along with the sperm. These accessory proteins cause female harm and are a major target of sperm competition. Forced monogamy typically leads to the an increase in female fitness. In beetles, where outcomes of competition are primarily decided by testis volume, there is little evidence of mate harm. In fact, polygamous females may live longer than monogamous males, stressing the importance of collecting data from multiple species.

1.4 Experimental evolution of anti-predator behavior

Predation has also been shown to be a powerful system in which to study behavioral evolution. It had been observed that there was significant color variation among males in populations of guppies (*Poecilia reticulata*) that experienced different levels of predation. Observations showed that conspicuous color spots seemed to lead to higher rates of predation. Endler (1980) tested whether predatory selection was directly responsible for the patterns of variation observed among these pools by constructing experimental ponds in the lab in which fish would be subjected to high and low levels of predation against a specific gravel background. They also transferred drab guppies from from a high predation pool to one lacking their primary predator and allowed them

to evolve for several years.

They found that all lab populations showed an increase in the number and size of spots until the introduction of predators, at which time, populations at low predation intensity continued to show increases in the size and number of spots in line with the controls. However, populations at high density showed a dramatic reduction in the size and number of spots. Also as predicted, the guppies introduced into low predation pools showed increases in spot number and size, in line with that observed in other natural populations with identical predators. This system has been expanded for the past several decades to continue to investigate the evolution of life history (Reznick and Bryga, 1987), the effects of alternate predators (Rodd and Reznick, 1991), the effects of resource availability (Grether et al., 2001), and behavior (Botham et al., 2006).

Similar to the studies described above, I have used experimental evolution to develop a set of populations in order to test hypotheses and predictions derived from evolutionary theory. I initiated replicate populations of *Drosophila melanogaster* that were subjected to episodic predation each generation by nymphs of the Chinese mantid (*Tenodera aridifolia sinensis*), along with respective predator free controls, that have been evolving for over 100 generations. The predator-evolved populations have shown an increase in viability that appears to be mediated by a combination of behavioral and morphological traits. These populations have allowed me to utilize an integrative approach to the study of evolution that included behavioral observation, morphological quantification, and statistical estimation of selection.

In Chapter 2, I discuss an experiment in which I address the long term predictive power of selection analysis. Before beginning experimental evolution, selection on wing size and shape was measured in the population that served as the base for the predation and control populations. After 30 generations of evolution, I remeasured selection on size and shape in the evolved populations. I then compared the actual trajectory of evolution to the trajectory predicted by selection on the base population. I also compare measures of selection from before and after evolution along with the variance in each population in relation to survival. I show that support for the predictions varies and discuss the relevant conclusions.

In Chapter 3, I discuss, the results from a comparative experiment performed on the experimentally evolved populations described above, which undergo episodic selection each generation, and another set of populations that undergo continuous selection in overlapping generations. I tested the prediction that variable selection leads to an increase in plasticity for traits that increase predation risk and that continuous selection leads to a loss of plasticity. I show that my data shed light on potential reasons for the mixed history of the risk allocation hypothesis. In particular, I focus on the need to understand the evolutionary history of the populations being tested as well as a more complete understanding of their behavioral repertoire.

In Chapter 4, I investigate the associations among predator avoidance, escape behavior, and morphology. Results presented in Chapter 2 suggest that changes in wing size and shape in the predator-evolved populations may have been correlated responses due to selection on other traits. Preliminary observations suggested that behavioral traits were likely candidates. I show that, though we cannot determine the exact targets of selection, there appears to be a correlation between escape behavior and morphological scaling relationships that merits further study.

Experimental evolution often leads to unexpected results, as it has in my work. However, these are fruitful avenues for further study because they allow us to investigate relationships that we would not have predicted. Testing theory is only a part of the goals for an experimental evolution program. Understanding the mechanisms and processes at work is a vital next step once the broader patterns have been identified.

CHAPTER 2

ADAPTATION TO A NOVEL PREDATOR: HOW WELL ARE WE ABLE TO PREDICT EVOLUTIONARY TRAJECTORIES?

2.1 Abstract

Evolutionary theory is sufficiently well developed to allow for short-term prediction of evolutionary trajectories. In addition to the presence of heritable variation, such prediction requires knowledge of the form of natural selection on relevant traits. While many studies estimate the form of natural selection, few subsequently examine the degree to which traits evolve in this direction. In this study we examined the form of natural selection imposed by mantid predation on wing size and shape in the fruitfly, *Drosophila melanogaster* on naive populations. We then evolved replicates of this population under predation pressure by these mantids for 30 generations, and examined the extent to which wing size and shape have responded in the direction predicted by selection on the base population. We demonstrated that wing form partially evolved along the predicted vector of selection for predator-evolved populations than for control lineages. In addition, we observed that the magnitude of selection on wing size and shape was diminished in populations evolving with mantid predators, while the direction of the selection vector differed from that of the ancestral population for shape. We discuss these findings in the context of the predictability of evolutionary responses, and the need for fully multivariate approaches.

2.2 Introduction

Biologists measure natural selection to help identify agents of selection, to infer how current phenotypes were influenced by past selection, to predict future evolutionary outcomes, and to study the process of adaptation. Since the publication of the seminal work by Lande and Arnold (1983), considerable effort has gone into measuring the form, magnitude and variability of phenotypic se-

lection (Hoekstra et al., 2001; Kingsolver and Diamond, 2011; Kingsolver et al., 2001; Morrissey and Hadfield, 2012; Siepielski et al., 2009). However, additional factors influence the trajectory of evolution such as the stability of the selective function and the genetic architecture of the traits (Agrawal and Stinchcombe, 2009; Hansen and Houle, 2008; Kirkpatrick, 2009), making such predictions difficult in practice. In this study, we address this predictability, by investigating the extent to which experimental populations of *Drosophila melanogaster* subject to predation risk, evolve along the trajectory predicted from phenotypic selection.

Studies have investigated how closely populations evolve along the direction predicted from the multivariate breeders equation, (as a function of both selection and the genetic variance-covariance matrix) (Agrawal and Stinchcombe, 2009; Blows et al., 2004; Hansen and Houle, 2008; Higgie and Blows, 2008; Hunt et al., 2007; Mcguigan et al., 2005; Schluter, 1996; Simonsen and Stinchcombe, 2010; Walsh and Blows, 2009). Yet in only a handful of cases has selection been observed for a sufficient amount of time (beyond a few generations) to evaluate these predictions.

Furthermore, the ecology and natural history of many organisms limits us to estimating phenotypic selection, generally over just a few generations (but see Grant and Grant, 2002, 2006; Ozgul et al., 2009). For some selective agents like predation, the organism is consumed, prohibiting (at least in the field) the measurement of many traits that are targets of selection. As a result, it may be challenging to predict the evolutionary trajectory of some traits involved with anti-predator activity. This might suggest a pessimistic view of our ability to predict the selective response in natural systems.

Despite these issues, convergent and parallel evolution are often observed among populations, suggesting that persistent and predictable selection may be relatively common (Conte et al., 2012), even if it is difficult to measure. Though estimates of the strength of selection via viability suggest it may be weaker than for other fitness components (Ajie et al., 2007; Hoekstra et al., 2001; Lind and Cresswell, 2005), repeated evolution of similar morphologies in response to predation upon several fish species (Dayton et al., 2005; Langerhans et al., 2004; Langerhans and Makowicz, 2009; O'Steen et al., 2002) suggests a strong and consistent regime of selection. Similar results have also

been observed for shell morphology among populations of snails in apparent response to predation (Auld and Relyea, 2011; DeWitt et al., 2000, 1999). When selection is relaxed by the removal of predators, even for just a few generations, trait means have been shown to change dramatically (Reznick and Ghalambor, 2005; Reznick et al., 1990, 1997), consistent with predation maintaining trait values in the face of potentially antagonistic selective effects. The prevalence of diverse, and often costly, traits that mediate interactions with predators suggests that predation profoundly influences fitness.

In this study we investigate how multivariate wing form of *Drosophila melanogaster* evolves along the trajectory predicted by initial estimates of phenotypic selection in response to predation by mantid nymphs (*Tenodera aridifolia sinensis*). This novel experimental system has a rather rare (but see Kuchta and Svensson, 2014; Svensson and Friberg, 2007) and useful attribute in which the wings are not consumed when the fly is captured by its mantid predator (Figure A.1). This allows us to collect the wings from both surviving and consumed flies to estimate the form and magnitude of natural selection on both size and shape. Multivariate shape provides a robust framework for evaluating evolutionary predictions. Size varies in one dimension, so wings can only become larger or smaller. Because of the high dimensional representation of shape, there are multiple ways in which shape can vary making it less likely to change in the direction of selection by chance alone. This enables us to make clear quantitative comparisons of the degree of similarity between predicted and observed response to selection (Pitchers et al., 2013). It also extends a well developed genetic system for use in studies of predator-prey interactions.

Wing size and shape in *Drosophila* have been used as a model system for evolution (Gilchrist and Partridge, 1999; Gilchrist and Huey, 2004; Gilchrist et al., 2004; Huey et al., 2000; Mezey and Houle, 2005; Pitchers et al., 2013; Weber, 1990*b*), genetics, and development (Dworkin and Gibson, 2006; Houle and Fierst, 2013; Palsson and Gibson, 2000). There is substantial segregating variation for wing size and shape, with some variants mapped (Dworkin et al., 2005; Mckechnie et al., 2010; Mezey et al., 2005; Palsson, 2004; Palsson et al., 2005; Weber et al., 1999; Zimmerman et al., 2000). Studies have demonstrated that genetic variation is available along many dimensions

of wing shape (Mezey et al., 2005). Using artificial selection, it has been demonstrated that this variation can be selected upon (Houle et al., 2003; Palenzona and Alicchio, 1973; Rochetta and Palenzona, 1975; Weber, 1990*a*,*b*, 1992). Yet little is known about the selective agents influencing variation in wing form (but see Hoffmann et al., 2007; Menezes et al., 2013) or the potential functional role wing form plays in avoiding predation.

We quantified the magnitude and direction of selection on wing size and shape in the outbred population that served as the base for our experimentally evolved populations. We then allowed replicates derived from this population to experimentally evolve either under episodic selection from a novel mantid predator or under predator-free conditions. In the evolved populations we quantified changes in wing form, with particular focus on the direction of change, relating it to the vectors of phenotypic selection predicted from the base population. We demonstrate that, although evolution of wing shape in the predator-evolved populations was more aligned with the initial vector of selection than that of the controls, not all of the change was in the direction predicted by the initial vector of selection. In addition, despite evidence for relatively consistent negative directional selection on wing size, we observed divergence in size between the predator-evolved populations. Furthermore, we measured phenotypic selection on the evolved populations, and demonstrate that the magnitude of selection on both size and shape is substantially diminished in the populations exposed to predation and is distinct from the initial vector of predicted selection. We discuss these results within the context of how populations change along a fitness surface, the importance of unmeasured traits and the degree of repeatability to agents of selection.

2.3 Materials and Methods

2.3.1 Base populations

We used an advanced intercross with 100 inbred lines to generate a synthetic outbred ancestral population referred to as the base. The inbred lines were derived from two populations of wild *Drosophila melanogaster* collected in fruit orchards in Maine and North Carolina (Goering et al.,

2009; Reed et al., 2010). Flies were round robin intercrossed for three generations and then allowed to mate randomly for 5 generations. We chose this approach, as a compromise to minimize confounding laboratory adaptation while still incorporating genetic variation present in natural populations. With this approach linkage disequilibrium among variants will likely be more extensive than in wild-caught flies. Post-intercross, we maintained the population at large size on cornmeal molasses media with live yeast in four 200ml culture bottles at 24°C and 60% humidity.

2.3.1.1 Predation environment

We used first instar nymphs of the Chinese mantid (*Tenodera aridifolia sinensis*) as predators. We ordered mantid egg cases from garden suppliers (Nature's Control Medford, Oregon) and supplemented these with egg cases collected locally from old fields in southern Michigan when necessary. We hatched and maintained egg cases at 24°C and 60% humidity. Approximately 100–400 mantids emerged from each egg case and were used as predators for the duration of the first nymphal instar. After hatching, we housed mantids at 18°C and 60% humidity in 710mL plastic cups with a mesh covered window for air flow. We placed a tissue at the bottom of each cup to trap moisture when watering to help maintain humidity. We also added a green plastic aquarium plant to provide substrate for mantids to perch upon. Unless otherwise specified we used five mantids per cup. These cups served as arenas used for experimental evolution and measuring selection as described below.

All episodes of predation occurred at 18.5°C and 60% humidity, and were initiated between 12-3 pm. We fasted mantids for 24 hours before each episode of selection to increase predation rates. Arenas were cleaned with 70% ethanol and water before use. 25 flies were introduced into each arena via a funnel, after which arenas were returned to the incubator. After 24 hours, all predation arenas were moved to a 4°C refrigerator to knock down flies and mantids to aid collecting. We then removed the mantids from each container, and surviving flies were censused and collected. When measuring selection, suriving flies and wings from dead individuals were placed in 70% ethanol for dissection.

2.3.2 Testing the role of flight in the escape response using a *vestigial*^{1} mutant population

To test whether flight played a role in the escape response, we tested whether wing loss would negatively impact survival under risk of predation. We introgressed a mutation in the *vestigial* (vg^1) gene into the base population by repeated backcrossing for 10 generations.The vg^1 mutation causes a nearly complete loss of the wing blade and associated musculature (Sudarsan et al., 2001). We competed individuals from the vg^1 mutant population with their wild-type conspecifics by placing 13 mutant and 13 wild-type flies in each of 16 arenas (8 arenas each for male and female flies). The survivors for both vg^1 and their wild-type conspecifics were counted after 24 hours with the predators.

2.3.3 Assaying phenotypic selection: base population

To assess how naturally segregating variation for wing size and shape might be associated with survival during predation events, flies from the base population were exposed to predation. Predation on males and females was assayed in separate arenas so that we could examine independent effects of sex. We placed 20 flies into each arena (9 arenas for each sex). Four days later we set up a second block of arenas (10 arenas for females, 8 for males). After predation, we collected all surviving flies and all wings from consumed flies from the bottom of the arenas. We also collected 100 individuals of each sex that were not exposed to predators. All bodies and wings were preserved in ethanol for dissection and measurement.

2.3.4 Experimental evolution

We randomly selected five hundred flies from the base population and used these as parents to generate the four populations for experimental evolution. We randomly assigned offspring of these 500 individuals to each of the treatments, with blocks of offspring for the different replicates. The predator free populations controled for selection and adaption independent of the predators (i.e. alterations in competitive environment). Selection was administered in two replicate sets each

consisting of one predation and one control population, hereafter referred to as PredR1, ConR1, PredR2, and ConR2. We offset the generational cycle of replicate 2 from replicate 1 by 2–5 days for logistical reasons, but the populations were otherwise treated identically. Each population was reared in four bottles with approximate densities of 100-500 eggs per bottle each generation. We did not explicitly control for density, but restricted egg laying time to 2–6 hours to avoid larval overcrowding. Bottles were reared at 24°C and 60% humidity until eclosion of adults.

Three days after eclosion of the first flies, progeny from each population were lightly anesthetized using CO_2, placed randomly into vials, and maintained at 18.5°C and 60% relative humidity. The following day, flies from a given treatment were mixed under anesthesia, to minimize inadvertent selection on developmental time. Flies (25/vial) were transferred to fresh vials, corresponding to the number of arenas used for predation in that generation. Each generation we varied the total number of arenas depending on the voracity of the current batch of mantids in order to ensure that the total number of survivors was large enough to limit the effects of drift (between 150–400 surviving individuals/generation). Flies from the control vials were similarly mixed under anesthesia after which we placed 50 flies into each of 8 vials. Remaining predation and control flies were set aside as backups. All flies were then returned to the incubator at 18.5°C and 60% for at least 24 hours prior to the episode of predation. Because egg cases were seasonally available, we occasionally used second instar mantids to maintain experimental evolution. However, second instar mantids were not used for any experimental trials. Control arenas were identical to predation arenas, only lacking mantids.

After selection, we collected all survivors from the predation arenas. To maintain similar population sizes we selected individuals at random from the control populations matching the number of male and female survivors from the respective predation population. All survivors were collected and placed into a single bottle for 24 hours. Every 24 hours, the survivors were transferred to a new bottle for a total of 4 bottles. After early indications that this prcedure was causing selection for early development, we changed the way laying procedure so that surivivors had access to all four bottles immediately after collection. After generation 17, all individuals from each treatment were

transferred into separate 32.5x32.5x32.5 cm Bugdorm-43030 polyester mesh cages, and allowed to recover for 30–45 minutes before four fresh bottles of food media were placed into each cage. After allowing sufficient time for egg laying, the bottles were then removed from the cages and placed at 24°C and 60% humidity. After breeding, remaining adult flies were stored in ethanol at -20°C. The approximate generation time for these populations was 17 days.

2.3.5 Assaying phenotypic selection: evolved populations

To examine how the fitness function changed as a result of experimental evolution, we repeated phenotypic selection (as described above) during generations 31 and 32 of the experiment. Given the large size of this experiment, it was performed in four blocks, with two blocks for each generation. At generation 31 of experimental evolution, we set up 14 arenas each of PredR1 females & males and ConR1 females & males. Five days later, we set up 14, 14, 8, and 9 arenas for PredR2 females & males and ConR2 females & males respectively. At generation 32, we set up 14 arenas each of PredR1 females & males and ConR1 females & males . Five days later we set up 13, 13, 14, and 14 arenas for PredR2 females & males and ConR2 females & males respectively. As before, we collected all surviving flies and unconsumed wings and stored them in ethanol. Overlapping egg cases were used for this experiment, and egg case of origin was used as a covariate in the model (see below). We distributed mantids so as not to confound predation effects across replicates and treatments.

2.3.6 Wing measurement & statistical analysis

Both wings from surviving flies were dissected and mounted on slides in 70% glycerol. Although most wings from dead individuals were collected as singly, on occasion, wings from dead flies remained connected to a remnant of the thorax. When this occurred, both wings from the dead individuals were mounted. Wings were also dissected from 25 flies that were stored from the initial generation of experimental evolution, and from every 10 generations following up to generation 50 to estimate the trajectory of size and shape change. Wings were imaged at 40X magnification

on an Olympus DP30BW camera mounted on a Olympus BX51 microscope using 'DP controller' V3.1.1 software. All images were saved in greyscale as TIFF files.

To capture landmark and semi-landmark data we followed a modified protocol (Pitchers et al., 2013) for the use of the WINGMACHINE software (Houle et al., 2003). We used the program TPSDIG2 V2.17 (Rohlf, 2010) to manually record the coordinates of two starting landmarks, and used WINGMACHINE to fit nine B-splines to the veins and margins of the wings in the images. We extracted 14 landmark and 34 semi-landmark positions, and performed Procrustes superimposition (Zelditch et al., 2012). After superimposition, the positions of semi-landmarks were allowed to slide along each segment of the wing margin/veins, minimizing Procrustes distance, using CPR V0.2 (Marquez, 2010). The data were checked for visual outliers at multiple stages; and putative outlier images were reexamined and splines re-fit if necessary. The (semi-)landmark configurations for all wings measured for this study were superimposed together, resulting in a common shape space. Centroid size was used as measure for size (Zelditch et al., 2012). For flies with both wings collected, we calculated the mean shape and centroid size per individual.

2.3.7 Model selection

For the univariate analyses, we evaluated model fits using Akaike's Information Criteria (AIC) and Bayesian information criteria (BIC). AIC has been shown to often 'prefer' more complex models than there was actually support for, particularly when sample sizes are large (Grueber et al., 2011). As a result, we used model weights from BIC throughout for consistency to perform model averaging when appropriate. Unless otherwise noted, all further analyses were conducted in R V2.15.1 (R Core Team, 2012).

2.3.7.1 Analysis of survival

For the vg^1 mutant and wild-type competition assays, we fit the model:

$$WT_{prop} \sim N(\mu + \beta_{sex}, \sigma^2) \tag{2.1}$$

where WT_{prop} was the proportion of wild-type survivors in each arena, and β_{sex} was the model coefficient for sex.

For the base and evolved populations, we measured survival ability as the total number of surviving flies in each container. For the base population, we fit the model:

$$Surv \sim N(\mu + \beta_{sex}, \sigma^2) \tag{2.2}$$

along with a set of expanded and restricted models (Table A.1) where *Surv* was the number of surviving flies in each arena and β_{sex} was the model coefficient for sex. Model averaging produced coefficient estimates indistinguishable from the model with best support so this model was used for further inference.

For the evolved populations we fit the model:

$$Surv \sim N(\mu + \beta_{SR} + \beta_{gen} + \beta_{eggcs}, \sigma^2) \tag{2.3}$$

along with a set of expanded and restricted models (Table A.2) where *Surv* was the number of surviving flies in each arena and β_{SR}, β_{gen}, and β_{eggcs} were the model coefficients for selection regime, generation of selection when the assays were performed, and the egg case of origin for the mantids in each arena. The coefficient estimates produced from model averaging models 1 and 2, which accounted for 95% of the overall model weighting, were indistinguishable from the model with best support so for simplicity we used it for further inference. For the above models, we confirmed the effects using generalized linear models (poisson with log link), or a logistic regression with similar results.

2.3.7.2 Analysis of phenotypic selection on size

We used the Lande and Arnold (1983) approach to examine selection acting on wing size in the base and evolved populations. As recommended by Janzen and Stern (1998), we used logistic regression on survival for statistical inference and general linear model on relative fitness to estimate coefficients. Relative fitness for each individual was calculated by scaling survival (0 for dead and

1 for survived) by the total proportion of survivors in each experiment. To measure linear selection in the base population we fit the model:

$$pr(W) \sim bi(p = logit^{-1}(\beta_0 + \beta_{size})) \tag{2.4}$$

along with a set of expanded and restricted models (Table A.3). W was absolute fitness (survival) and β_{size} was the model coefficient for standardized wing centroid size. The coefficients produced by model averaging were indistinguishable from the model with best support so we used the model with best support for further inference. It should be noted that we are estimating the linear S, and non-linear C selection differentials (Brodie III et al., 1995). The β's in the equations are used to represent estimated model parameters, and do not represent selection gradients.

To measure linear selection in the evolved populations we fit the model:

$$pr(W) \sim (p = logit^{-1}(\beta_0 + \beta_{size} + \beta_{SR} + \beta_{rep} + \beta_{sex} + \beta_{gen} + \beta_{size\times SR} + \beta_{rep\times gen} + \beta_{sex\times gen} + \beta_{SR\times rep})) \tag{2.5}$$

along with a set of expanded and restricted models (Table A.4) where W was fitness and β_{size}, β_{SR}, β_{rep}, β_{sex}, and β_{gen} were model coefficients for standardized wing centroid size, selection regime, replicate, sex, and the generation of experimental evolution respectively. We fit separate models as above including the quadratic effect of size to estimate non-linear selection. Estimates for non-linear selection on size were near zero, non-significant, and did not improve model fits in either the base or the evolved populations.

Non-parametric estimation of the form of the fitness functions substantially aids visualization and interpretation of fitness functions (Schluter, 1988). We therefore used generalized additive models from the the MGCV package V1.7.22 (Wood, 2004) to fit cubic splines to subsets of the data from each experiment corresponding to the relevant significant effects estimated by the logistic regression analyses. Optimal smoothing parameters were estimated using the REML method.

2.3.7.3 Variance in size & shape

One additional approach to investigating natural selection is to examine the changes in phenotypic variance before and after the selective event (Endler, 1986). Under either directional or stabilizing selection, a reduction in variation would be predicted. Under disruptive selection however, we would predict an increase in variation. Analyzing differences in variance between dead and surviving flies, as well as between predation and control populations, may therefore provide additional information on the type of selection occurring in these populations. We used Levene's test to assess changes in variance for wing size, using deviations from the median rather than the mean since this approach is more robust to departures from normality. For the base population, we modeled the main effects of sex and size because our previous analyses lacked support for an interaction between sex and the form of selection. We fit the model:

$$Ld \sim N(\mu + \beta_{sex} + \beta_W, \sigma^2) \tag{2.6}$$

where Ld was the Levene's deviates for each individual, β_{sex} was the model coefficient for sex and β_W is the model coefficient for absolute fitness. Though our previous analyses do not suggest that selection acting in the evolved populations differed between replicates, differences in size between PredR1 and PredR2 suggest that its inclusion is appropriate. We fit the model:

$$Ld \sim N(\mu + \beta_{SR} + \beta_{rep} + \beta_{sex} + \beta_W + \beta_{SR\times rep}, \sigma^2) \tag{2.7}$$

where Ld was the Levene's deviates for each individual and β_{SR}, β_{rep}, β_{sex}, and β_W were the model coefficients for selection regime, replicate, sex, and absolute fitness, respectively. Confidence intervals for all estimates were generated by non-parametric bootstraps, in order to avoid issues with non-normality of residuals. We also calculated the coefficient of variation for each of the groups modeled above for visualization because it normalizes for differences due to sexual size dimorphism as well as size differences among the base and evolved populations.

To compare levels of variation in shape we took a somewhat simpler approach. We expressed

the variability of each group as the trace of its covariance matrix for shape. We then bootstrapped the data to generate samples of each covariance matrix in order calculate confidence intervals on the estimated matrix trace. Non-overlapping (95%) confidence intervals intervals were then used to infer statistical support for differences in variance among groups.

2.3.7.4 Multivariate analysis of shape

In our initial analyses, we found that the modeled effects of allometry and sexual dimorphism were extremely consistent between treatments and over time (i.e. the vectors of model coefficients for sex and for size were very tightly correlated; see below). In order to facilitate the interpretation of the modeled coefficients of selection and generation number, we therefore sought to exclude these effects from our analyses. With data from all wings pooled, we fit the model:

$$\mathbf{S} \sim N(\mu + \beta_{sex} + \beta_{size}, \Sigma) \tag{2.8}$$

where $\mathbf{S}$ is the matrix of Procrustes coordinates and β_{sex} and β_{size} are the vectors of model coefficients for sex and for wing centroid size respectively. Σ is the "error" covariance matrix. We retained the residuals from this model as our shape variables.

Configurations of Procrustes coordinates by definition include dimensions without variance. The Procrustes superimposition results in a deficiency of 4 ranks (1 each for removed size and rotation information, and 2 for position), and each semi-landmark may contribute as little as 1 added dimension (Zelditch et al., 2012). In order that the shape data would not be rank deficient, we extracted principal components from the (96-dimensional) residuals, and retained the first 58 principal components, comprising $> 99.9\%$ of the shape variance in the full set of residuals. Removing variation due to location, rotation, and size led to the loss of 4 dimensions, and 34 additional dimensions were lost because, even though the sliding landmarks have two measured coordinates, they only vary in one dimension. Shape PC's used in all of the analyses below are thus of full rank, and are expressed in a common sub-space.

2.3.7.5 Modelling shape change

Separately within each of the four evolved populations, we estimated the direction of observed evolutionary change as the vector of model coefficients from the multivariate linear model:

$$\mathbf{S}_p \sim N(\mu + \beta_{gen}, \Sigma) \tag{2.9}$$

where $\mathbf{S}_p$ is the matrix of principal component scores for shape in a given population and β_{gen} is vector of model coefficients for time, expressed as the number of generations removed from to the base population. Once we had estimated these vectors of parameters, we compared their directions by calculating vector correlations as:

$$r = \frac{|\mathbf{a} \cdot \mathbf{b}|}{\|\mathbf{a}\| \times \|\mathbf{b}\|} \tag{2.10}$$

where $|\mathbf{a} \cdot \mathbf{b}|$ is the absolute value of the dot (scalar) product between vectors $\mathbf{a}$ and $\mathbf{b}$, while $\|\mathbf{a}\|$, and $\|\mathbf{b}\|$ are the magnitudes (L^2, or Euclidean norm), for each vector. The absolute value for the dot product was used to avoid any numeric issues with arbitrary sign changes that can occur computationally (during the bootstrapping procedure, see below). Thus $r = 0$ represents no similarity between the vectors while $r = 1$ means the two vectors point in an identical orientation (but possibly opposite in direction). Given that r is a multivariate extension of the Pearson correlation co-efficient ρ, we consider this a more intuitive measure than the vector angle ($\theta = \arccos(r)$ in radians) which has been used elsewhere. Confidence intervals were computed using non-parametric random pairs bootstrapping, from 10,000 bootstrap iterations. This approach was used both to compare the direction of $\mathbf{S}$ as measured in all five populations, and to compare the directions of observed shape change among the evolved populations.

To illustrate the magnitude of change in wing shape during experimental evolution, we calculated a shape score (Drake and Klingenberg, 2008). Briefly, we projected the shape data onto a line in the direction defined by the vector of model coefficients for the generation term (β_{gen}) from model (3):

$$\textbf{shapescore} = \mathbf{Y}\beta^T(\beta\beta^T)^{-0.5} \tag{2.11}$$

The shape score provides a univariate measure of shape change that can be plotted against generation number to visually assess the magnitude and linearity of the relationship (Drake and Klingenberg, 2008). We used custom R functions to calculate vector correlations and shape scores.

2.3.7.6 Selection on shape

Within each population, we estimated the vector of linear shape differentials (**S**). Traditionally this would be calculated as vector of differences between the mean phenotype of survivors and the mean phenotype of those individuals that were preyed upon. Here we estimated **S** using a 2-block partial least squares (PLS) approach (Gomez et al., 2008, 2006; Klingenberg and Zaklan, 2000; Klingenberg and Monteiro, 2005; Mitteroecker and Bookstein, 2011; Rohlf and Corti, 2000) with the matrix of the 58 shape PC's forming one block, and the vector of survival data (0 or 1 for dead or survived) as the second block. We note that in this case this estimate of **S** is proportional to the differences between the mean shape configurations for the dead and survivors.

It is important to note that wing shape itself is the trait, and not individual landmarks/PC's. After Procrustes superimposition, individual landmarks and semi-landmarks cannot be meaningfully interpreted independent of the whole shape configuration and the superimposition can generate correlation between landmarks that is confounded with biological correlations (Zelditch et al., 2012). Thus, interpreting the selection gradients, β, from a multiple regression for shape (*sensu* Lande and Arnold, 1983) for "individual" shape variables is biologically meaningless (Albert et al., 2008). In addition, selection gradients can be difficult to visualize for shape (Klingenberg and Monteiro, 2005; but see Mitteroecker and Bookstein, 2011), in particular because estimating the inverse of the phenotypic covariance matrix, $\mathbf{P}^{-1}$, can be problematic. Indeed, upon resampling, we observed computational difficulties due to a lack of stability in the estimation of $\mathbf{P}^{-1}$. One alternative is to retain only the first few PC's and analyze them as if they were independent traits (Gomez et al., 2008, 2006; Kuchta and Svensson, 2014). This is still sub-optimal, however, since substantial vari-

ation and selection may be missed and the biological interpretation of any selection that is detected is difficult. While this is an important and outstanding issue, we elected to use selection differentials for the shape analyses because they retain biological meaning and the focus of the study is on the predictability of selection not its specific form or estimation. However, this does mean that the results need to be interpreted as a combination of both direct and indirect selection on shape.

We estimated total selection on wing shape as the magnitude of the vector of the selection differentials, $\|\mathbf{S}\|$, and used sampling with replacement of the data to generate non-parametric bootstrap confidence intervals on these estimates. Additionally, we permuted survivorship relative to the measures of shape to assess the null hypothesis that wing shape does not contribute to variation for survivorship. We also compared the directions of the **S** vectors using vector correlations as described above. Finally, we wanted to assess the degree to which the experimental evolution populations had evolved in the direction 'predicted' by selection as measured in the base population. To do this we calculated the vector correlations between the **S** vector measured in the precursor population and the vector of model coefficients for generation (β_{gen} from model (8)) as modeled separately for each population.

2.4 Results

2.4.1 Evidence that flight aids in the escape response

To test whether flight performance and wing form were potential targets of selection driven by the mantid predators, we introgressed a mutation in the *vestigial* (*vg*) gene into our base outbred population that nearly completely ablates the wing blade and associated flight muscles. We competed vg^1 (functionally wingless) flies and their wild-type conspecifics with the predators. As predicted, the vg^1 individuals were disproportionately the targets of predation. The survivors for both sexes consisted of approximately 60% wild-type and 40% mutant individuals (Figure A.2), consistent with a role for flight and possibly wing morphology in the escape response of *Drosophila*.

2.4.1.1 Predator driven selection on natural variation for wing form

We next asked how natural variation for wing form was associated with survivorship by exposing flies from the base population to the mantids. We observed evidence for significant negative directional selection on wing size (Figure A.3B, Table A.1, S = -0.29 $\pm$0.11, p $\simeq$ 0.01) with little evidence for nonlinear selection (C = -0.05 $\pm$0.22, p $\simeq$ 0.16). Visualization by fitting cubic splines to the survival data (Schluter, 1988) was consistent with the estimates of directional selection (Figure A.3A). Despite sex specific differences in survivorship (6.7 $\pm$0.75 & 3.8 $\pm$1.07 survivors per arena for females and males, respectively), evidence was weak for an interaction between selection on size and sex. However, we do not know the relative contributions of direct selection on wing size and indirect selection on other traits to the selection differential we have estimated.

Additionally, shape has been shown to be correlated with escape ability in other animals (Dayton et al., 2005; Langerhans et al., 2004; Langerhans and Makowicz, 2009; Svensson and Friberg, 2007). We used partial least squares (PLS) to estimate the vector of selection differentials for shape (**S**) in the base population. We visualized the vector, **S**, for shape comparison. Figure A.3B showed selection for a change in aspect ratio in which inividuals with relatively longer and narrower wings had higher fitness.

2.4.2 What does wing form look like after experimental evolution?

As expected, survival in both predator populations increased, compared to the controls (10.86 $\pm$0.78 & 13.98 $\pm$0.79 survivors per arena for control and predation populations, respectively; Figure A.4A). This represents a $\sim$30% increase in survivorship relative to the control populations. We did not observe differential survival between the sexes in this experiment for either selection regime.

To track changes in wing form, we measured individuals stored during the experimental evolutionary process every ten generations, from the base to generation 50. Wing size of all populations increased $\sim$3.7% over the 50 generations of experimental evolution (0.0035 mm $\pm$0.0004 mm per generation). This change was most likely a result of selection due to non-predatory aspects

of the experimental evolutionary procedure. Though highly variable, average effective population size was large enough to counter the effects of drift (effective size = 245.33 ± 22.37 flies) and changed very little over the course of the 30 generations. In fact, both replicates showed a slight increase in effective population size (R1 = 0.9 ± 1.26 flies/generation, $p \simeq 0.48$; R2 = 1.71 ± 1.78 flies/generation, $p = 0.33$), though these differences were non-significant. In addition, all populations increased at similar rates and maintained the same overall sexual dimorphism. In particular, note the rapid increase between generations 20 and 30 (Figure A.5). This period corresponded to a change in the experimental procedure that corresponded to a relaxation of selection for early development and lower larval densities. However, environmental variation was relatively large in these samples collected directly from the experimental evolution regime.

To more carefully estimate size differences among the evolved populations, we measured wing size in the overall population by using the wings from the dead and surviving flies from the phenotypic selection experiments, as all flies were reared under density controlled conditions. Thus environmental and genetic effects were not confounded. Comparison of the number of surviving and dead flies from this assay to the number of wings recovered suggests that nearly all wings from dead individuals were recovered, and should provide reasonable estimates.

Under these conditions, the relevant contrast is the difference between the control populations and predation populations. We found that the two control populations had similar wing sizes, yet the two populations evolved under risk of predation diverged in size (Figure A.4B) even though all populations showed a general size increase relative to the ancestral population. Surprisingly only PredR2 has evolved smaller wings than the controls, $\sim$1%, as predicted by the estimate of negative directional selection on the base population while PredR1 evolved wings that were $\sim$2% larger.

In terms of shape, all four populations have evolved from the base population, though not to an equal extent. We visualized the evolutionary trajectories of the four populations by plotting the shape score for generational effects (equation 10) (Figure A.6A & B). In all four cases the evolutionary trajectories were best described by a simple linear model. Whereas the two control populations have changed in a very similar fashion (Figure A.6A), the two predation populations are

clearly divergent, with wing shape in PredR2 evolving significantly more rapidly than in PredR1 (Figure A.6B).

Over the course of experimental evolution the wings of all populations have changed aspect ratio: their length increasing slightly as their depth decreases. This change is most pronounced in PredR2 (Figure A.6C). Other than the differences in aspect ratio, PredR1 and PredR2 differ most noticeably in the response of the cross-veins and the distal end of L5. PredR1 demonstrates a proximal shift in the posterior cross-vein and an anterior shift in the attachment of L5 to the margin; by contrast in PredR2 there was no change in L5 and an anterior shift in the anterior cross-vein.

2.4.3 What do the fitness functions look like after experimental evolution

After 30 generations of experimental evolution, we again exposed flies that evolved with (and without) predators to a bout of predation. We observed negative directional selection for size in the control populations (S = -0.16 $\pm$0.07, p $\simeq$ 0.0001; Figure A.7A), consistent with the pattern of selection on the ancestral base population but half the magnitude. We also observed negative directional selection in the predation populations, but of diminished magnitude relative to the controls (S = -0.06 $\pm$0.03, p $\simeq$ 0.0005; Figure A.7B). Both the control and the predation populations showed extremely weak quadratic selection on size (C = 0.0001 $\pm$0.03, p $\simeq$ 0.73). We might reasonably expect that the reduction in the magnitude of directional selection in PredR2 was a result of evolutionary change in wing size in response to selection.

We assessed selection on wing shape in the evolved populations as $\mathbf{S}$; the vector of selection differentials between captured flies and survivors and compared the magnitudes of total selection on shape from the differential, $\|\mathbf{S}\|$. For the estimates of $\|\mathbf{S}\|$, we also generated distributions under the null expectation (of no association between wing shape and survival) using permutations of the data. In addition we also calculated the vector correlations between differentials in order to quantify their degree of alignment. For both approaches we computed confidence intervals on our estimates by applying a non-parametric bootstrap approach.

As can be seen from Figure A.7C, there was evidence for a significant association between

shape and survival in the presence of the mantid predators for all populations: the estimates of $\|\mathbf{S}\|$ exceeded the 95% threshold permuted under the null hypothesis. Also notable are the much smaller $\|\mathbf{S}\|$ estimates of the predation populations compared to that in the base population. This evidence is consistent with a relative reduction in the magnitude of selection experienced by the predation populations after 30 generations. Interestingly, there is some evidence for difference in $\|\mathbf{S}\|$ between the two control populations, however both still exceed the predation treatment regimes.

2.4.4 Has wing form evolved in the direction predicted by selection on the base population?

While there has been evolution of shape in all four populations, we wanted to assess how much of the observed change is in the predicted direction (based on **S** in the ancestral base population). We calculated the vector correlations between the generation shape change vectors from each evolved population and the **S** vector from the base population and observed that the evolutionary responses of the predation populations were more aligned with the predicted vector compared with the control populations (Figure A.8). It is notable that none of the populations are particularly highly aligned with the initial predicted vector.

Given that both predation populations have experienced a similar reduction in the magnitude of selection (as represented by $\|\mathbf{S}\|$: Figure A.7C), and a similar amount of evolutionary change in this direction (Figure A.8), it appears that they experienced similar changes in the selective function for shape, despite their divergent evolutionary response for size.. This suggests that the two predation populations are evolving different avoidance strategies in response to the predation pressure imposed by the mantids — likely involving traits other than wing morphology. In the case of PredR2 there is evidence that the reduction in the intensity of selection is associated with evolution in the predicted direction, but it seems likely that there may be other adaptions occurring in PredR1

2.4.5 Changes in variance in both size and shape

Changes in variation can also provide information about the form of selection experienced by a population. Evidence for differences in size variance between survival classes in the base population was weak (Levene's deviates = 0.28 $\pm$0.04, 0.27 $\pm$0.03, 0.24 $\pm$0.04 for unselected, dead, and surviving individuals respectively, $p \simeq 0.25$). Males had lower variance for all survival classes (-0.07 $\pm$0.03, $p < 0.001$). We did not find differences in size variance for the evolved populations, though the populations differed from one another. ConR1 and PredR2 having equal variance (Levene's deviates = 0.11 $\pm$0.01, 0.11 $\pm$0.01 respectively). ConR2 had higher variance (Levene's deviates = 0.15 $\pm$0.01), and PredR1 had lower variance (Levene's deviates = 0.10 $\pm$0.01). Surviving individuals trended towards lower variance, but the difference was not significant (-0.003 $\pm$0.006, $p \simeq 0.37$). Males again had lower size variance, but a much lower magnitude of differences (-0.015 $\pm$0.006, $p < 0.005$).

Estimates of variance for shape in each population (the trace of the covariance matrix) show a dramatic reduction in variance in surviving flies and lower overall shape variance in the populations that evolved under predation risk (Figure A.9). Not only is the variance lower in the predation populations as compared to the controls for shape, but the surviving populations have much lower variation when compared to the populations that were captured and eaten by the mantids suggesting that selection has already reduced variation in the evolved populations and continues to do so.

2.5 Discussion

For several decades, phenotypic selection analysis has been used to attempt to identify the primary targets of selection within natural populations under the assumption that the presence of selection on specific traits would provide information about about how those traits evolved and what future changes could be expected. The striking levels of convergence and parallellism in several well known study systems suggests that this assumption may be valid (Auld and Relyea, 2011; Dayton et al., 2005; DeWitt et al., 2000, 1999; Langerhans et al., 2004; Langerhans and Makowicz, 2009;

O'Steen et al., 2002). In this study we measured phenotypic selection on a naive population in response to a novel predator. We then re-measured the strength and direction of natural selection after populations were allowed to evolve under natural selection with the predator to determine whether our results would match the patterns of parallel response cited above. We found that the populations evolved divergent morphology for size but relatively consistent shapes. What do these results tell us about the form of natural selection, and what are the implications for its use in evolutionary prediction?

To use the breeder's equation to predict an evolutionary response, we require not only a vector of directional selection, but heritable variation along the same axis as selection (Hine et al., 2011; Walsh and Blows, 2009). The direction of this genetic variation is determined by the size and structure of the genetic covariance matrix **G**. A number of studies have demonstrated that populations tend to evolve along genetic lines of least resistance (Mcguigan et al., 2005; Schluter, 1996), not necessarily the direction of strongest selection.

Previous work has demonstrated that there is considerable segregating genetic variation in most populations for wing shape. In particular the effective dimensionality of **G** for wing shape is quite high, and close to the number of measured traits (Mezey and Houle, 2005). We did not attempt to estimate **G** for the base population we used. However, genetic variation among the progenitor strains used to generate the population shows a high effective dimensionality (data not shown), consistent with previous results from other populations. It is possible that genetic variation in the direction of selection imposed by mantid predation may be minimal, and that the genetic line of least resistance is not perfectly aligned with this direction. Thus at least some of the common changes in wing form may be the result of a combination of lab domestication and evolution along the genetic lines of least resistance. Despite this, we see clear evidence for more shape change in the predation regimes consistent with the initial vector of selection. Given the high dimensionality (58) of shape, this is a pronounced effect, demonstrating that even with potentially countervailing selective and genetic forces, selection is still altering shape as predicted.

In addition to the need for available genetic (co)variation, there are several factors that can

influence the evolutionary response to directional selection including: indirect selection due to correlated traits, stabilizing selection, fluctuating selection, and fitness trade-offs (Kingsolver and Diamond, 2011). The selection differentials reported include direct and indirect selection, so even though we cannot estimate the separate contributions of each, we saw significant total selection on both wing size and shape. Additionally, neither the base nor the evolved populations showed evidence for stabilizing selection that might have reduced the strength of directional selection.

2.5.1 How does the form of selection change after 30 generations of experimental evolution

The pattern of selection imposed by the mantids on size appeared to remain relatively stable over the 30 generations consistent with the conclusions reached by Morrissey and Hadfield (2012). Our measure of S in the ancestral base suggested strong directional selection for smaller wings ($\sim$-0.29). After 30 generations of evolution, the magnitude of S on the control populations had reduced in half (-0.16, Figure A.7), yet the directionality was the same. This reduction in the magnitude is, perhaps, unsurprising since the difference in sample size—nearly an order of magnitude greater for the evolved populations—allowed for more precise estimation. The estimate is also in line with the median reported by Kingsolver and Diamond (2011) for size traits ($|0.14|$), though larger than the mean for selection via viability ($|0.08|$). As a result, there is little evidence to suggest that temporal variation in the directionality of selection from generation to generation resulted in the divergence in size between the predation populations, particularly because we still observed evidence for selection for smaller wings.

2.5.1.1 Selection on shape

For shape, the picture is less clear than for size. Because of the high dimensionality of shape, not only is estimation much more difficult, but there is a much larger available phenotype space. Perhaps unsurprisingly then, the vector correlations between selection in the base population and the control populations are reasonably low ($\sim$0.35). The degree to which there was true variation in direction of selection for shape, as compared to estimation issues (even with our large sample sizes),

remains unclear. Indeed, this is one of the major reasons we used $\mathbf{S}$ instead of β, as estimating $\mathbf{P}^{-1}$ proved to be computationally difficult, and caused problems during resampling. Despite this, both PredR1 and PredR2 show considerable overlap between the vector of shape change during evolution and the direction of selection predicted in the base population ($r \sim 0.5$, Figure A.8). This suggests that even though the form of selection for shape is apparently less stable than for size, it has not resulted in substantial divergence between populations.

It is worth considering what is lost by using the selection differential $\mathbf{S}$ instead of the gradient, $\beta = \mathbf{P}^{-1}\mathbf{S}$. For most phenotypic selection studies the main difference relates to disentangling direct and indirect selection on traits (pre-multipling by $\mathbf{P}^{-1}$ removes the phenotypic covariation). Shape data is unique, in that the different variables are not independent traits. Instead the whole configuration (as represented by a vector for each individual) is a geometric representation of the shape "trait". Pre-multiplication by $\mathbf{P}^{-1}$ has the potential to change the observed orientation of the vector of the selection differential $\mathbf{S}$, however it also causes difficulties with interpretation of the resulting selection gradients, and so the preferred method is to visualize the selection differentials as we have done here (Klingenberg and Monteiro, 2005; but see Mitteroecker and Bookstein, 2011 for an alternative perspective). Other groups have instead utilized a small number of principal components of the shape data in a standard Lande–Arnold selection gradient analysis (Gomez et al., 2006; Kuchta and Svensson, 2014). However, this utilizes a fraction of the variation in shape, with no guarantee that it represents the components of variation under selection. Thus a full multivariate approach is needed (Klingenberg, 2010) though we currently lack an accepted standard *sensu* Lande and Arnold (1983). We suggest that continued effort and discussion into estimating and visualizing selection on shape, as well as determining the appropriate "dimensionality" of such effects is warranted.

2.5.2 Possible causes of divergence and parallel evolution

Unknown fitness trade-offs and lab adaptation may have played a role in the divergence in size between PredR1 and PredR2. During the course of evolution, all four evolved populations showed

a net increase in wing size of $\sim$3.2 % and a lengthening and broadening of the wing blade in direct contrast to the smaller, longer, and narrower wings favored by selection in the base population (Figure A.3). These changes were remarkably consistent among the four populations and are likely a result of selection due to shared aspects of the experimental evolutionary process independent of the predators. However, though the directionality of the shared evolved response and selection measured in the base population suggests that evolution may be slowed in the predation populations, this gives little indication as to why PredR1 diverged from the predicted size trajectory.

This divergence between PredR1 and PredR2 could have been caused by drift between the replicates over the 30 generations of experimental evolution. However, this explanation is unsatisfactory as the evidence of drift is missing in the control populations in which it would be expected to dominate. In addition, the effective population size of the evolved populations remained high enough throughout the 30 generations to make the fixation of alleles by drift alone unlikely (Figure A.10). In addition, other aspects of the selection procedure could have differed between PredR1 and PredR2. Though the utmost care was taken to control variation between the replicates, differences in the health and voracity of the mantids, as well as in some other environmental factors were unavoidable, possibly contributing to this effect.

Where do these results leave us? We possess robust theory for measuring selection, and for predicting evolutionary responses into the near future (Lande and Arnold, 1983). However, we are often left to assume that populations will evolve phenotypes in the distant future consistent with these estimates. Though a number of other researchers have examined the evolutionary consequences of manipulating predation regimes long term, notably the work of David Reznick and colleagues (Reznick and Ghalambor, 2005; Reznick et al., 1990, 1997), few studies have investigated how well evolutionary responses coincide with specific measures of selection. The *Drosophila*-mantid system described here allows us to maintain specific selective pressure in a relatively homogeneous environment on a population with a known history. This allows us to not only impose specific selection pressures, but to remeasure selection itself during the evolutionary process.

It is likely that unmeasured anti-predator behavioral traits played an important role in the di-

vergence between predation populations for both size and shape. Unmeasured traits that may be under selection (and genetically covary with measured traits) can profoundly influence the biological inferences we make about natural selection, and evolutionary response. While many studies of phenotypic selection attempt to examine multiple traits that mediate the ecological interactions that generate variation in fitness, it is impossible to capture all of them in any one study. In a system like ours, where we employed a novel predator for *Drosophila*, anti-predator behaviors that were initially rare in the progenitor population can rise in frequency, fundamentally changing aspects of selection on other traits. In particular, differences between the predation populations due to founder effects may have placed the predation populations on different trajectories early during the evolutionary response. For instance, if the escape response to direct attacks was the primary strategy for one population, but the ability to avoid the predators was important in the other, then the response to predatory selection might be different between the two populations as we have seen here. Study systems like the one used here allow for additional future work to address these questions in a relatively straightforward manner.

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CHAPTER 3

DOES VARIATION IN PREDATION RISK LEAD TO THE EVOLUTION OF PLASTICITY? A TEST OF THE RISK ALLOCATION HYPOTHESIS USING EXPERIMENTAL EVOLUTION

3.1 Abstract

The risk allocation hypothesis predicts the ways in which prey should flexibly adjust their behavior in response to temporal variation in predation risk. It states that as temporal variation in attack ratio between high and low risk increases, prey should perform more anti-predator behaviors when at high risk. It also states that as the proportion of time spent at risk increases, prey should invest less effort into anti-predator behaviors. Though the hypothesis sparked a flurry of research, support for the affects of risk allocation has been mixed. Tests of the hypothesis typically evaluate the conditioned rsponse of prey animals to predator cues. However, it is unclear the extent to which the prey animals studied correctly perceive the risk dynamics. Furthermore, the predicted reuduction in anti-predator behavior when a higher proportion of time is spent at high risk could simply be due to habituation to the predator cues. In order to untangle some of these complicating factors, we took a novel approach in which we evolved populations of *Drosophila melanogaster* in response to either variable or continuous predation by nymphs of the Chinese mantis (*Tenodera aridifolia sinensis*). We found that predation had significant effects on the evolution of courtship, anti-predator behavior, and overall activity, but not on aggression. Contrary to predictions, both the continuous and variable predation populations lost plasticity for courtship behavior, but consistent with predictions, only the episodic predation populations increased anti-predator behaviors. Though we found limited support for the predictions of risk allocation for specific types of behavior, our results suggest that evaluating a more complete behavioral repertoire will shed light on the complex ways in which prey can respond to variation in risk.

3.2 Introduction

The predation risk allocation hypothesis was proposed by Lima and Bednekoff (1999*b*) in response to the failure of behaviorial ecologists to incoporate the effects of risk on behavioral decision making into experimental and theoretical work. As a result they developed a model in order to formalize predictions regarding how prey should behave in response to temporal variation in predation risk. They concluded that as the risk of predation increased, prey should invest more effort into vigilance during those high risk situations thereby reducing risky behaviors (e.g. foraging). To compensate for the potential loss of resources during these high risk periods, during low risk situations prey should subsequently reduce vigilance and invest more effort into risky behaviors. In addition, they concluded that, as the proportion of time spent at high risk increased, organisms should decrease vigilance, especially during rare instances of low risk. This has been dubbed "the paradox of risk allocation" (Ferrari et al., 2009) due to the counterintuitive result that organisms experiencing a greater amount of risk should actually decrease anti-predator activity.

Many tests of the risk allocation hypothesis performed since the publication of Lima and Bednekoff (1999*b*) were reviewed by Ferrari et al. (2009) in order to evaluate the extent to which the predictions were supported by empirical data, primarily but not exclusively in aquatic systems. They found that evidence for risk allocation has been mixed with some studies showing complete support, some showing support for only a few predictions, and others showing no support at all (for details on individual studies, see Ferrari et al., 2009). A common divergence from predictions was that when risk was variable, prey organisms did decrease activity (or increase vigilance depending on the study) in response to predator cues, but did not show the predicted increase in activity when the predator cues were removed. Ferrari et al. (2009) suggested that this might be because the animals used in the study were not food stressed and therefore did not need to "make up" for a deficit during low risk situations. They further suggested that studies showing partial or lack of support may be due to the prey being unable to accurately estimate the risk environment imposed by the researchers and that longer exposure was necessary (as in Brown et al., 2006; but see Slos and Stoks, 2006).

Additional studies published after this review have attempted, to varying degrees, to address these concerns and have expanded the range of terrestrial systems being tested, including ungulates (Creel et al., 2008; Sönnichsen et al., 2013), reptiles (Martín et al., 2009), birds (Paclík et al., 2012; Rodriguez-Prieto et al., 2009; Thomson et al., 2011), rodents (Kotler et al., 2010; Suselbeek et al., 2014; Unck et al., 2009), and spiders (Sitvarin and Rypstra, 2012), along with additional tests in aquatic systems (Ferrari et al., 2010, 2008; McMahan et al., 2013; Salice and Plautz, 2011; Schoeppner and Relyea, 2009; Trussell et al., 2011; Wojdak and Trexler, 2010). Though general support for some aspects of risk allocation is stronger in these recent studies, few tested all aspects of the hypothesis simultaneously, and the results remain mixed.

The ability of prey to accurately assess the dynamics of the risk regime is a crucial unknown when evaluating the relative success of the risk allocation hypothesis. Most studies rely on the assumption that all prey can learn the structure of the risk regime imposed by the experimentors and flexibly adjust their behavior accordingly. Though it is certainly true that organisms are able to adjust their behavior to the current environment, it is not certain to what extent the structure of the risk regime that is perceived by the prey animals in these studies matches what was intended. To explore this, Ferrari et al. (2008) tested whether the predictability of the risk structure would affect prey behavior. They hypothesized that predictable risk would improve the ability of the prey to perceive high and low risk situations, yet they found no effect of variation in predictability on prey behavior.

Despite this, studies that have observed prey behavior in the wild under natural variation in predation risk do show strong support for many aspects of risk allocation (Creel et al., 2008; Kotler et al., 2010; Sönnichsen et al., 2013; Suselbeek et al., 2014). This may be due not only to the fact that prey in these studies experience relatively predictable variation in risk throughout a lifetime, but also because that variation has persisted for multiple generations. In fact, there is a growing appreciation for the importance of the evolutionary history of predation risk experienced by prey populations because of the ability of selection for particular behavioral patterns, such as those described by the risk allocation hypothesis, to increase fitness (Beauchamp and Ruxton, 2011;

Brown et al., 2009; Salice and Plautz, 2011).

In this study, we explore the effects of selection on risk allocation by taking a new approach in which we observe the changes in courtship, aggression, and anti-predator behaviors that occur after many generations of laboratory evolution under a novel predatory risk. Most previous studies have focused on the learned responses of prey to variation in predation risk. However, if risk allocation does indeed lead to increased fitness through behavioral flexibility, stable variation in predation risk should also lead to the evoluiton of these behavioral patterns. Here we describe an experiment in which we use populations that have been evolved under constant or variable predation risk in a consistent and predictable manner in the laboratory. As a result, we are able to test the responses of multiple prey individuals under real predation risk. Furthermore, because we are testing an evolved response to a particular risk regime, we can use naive prey, thereby avoiding the challenges of conditioning prey to a specific risk regime and confounding the response to the proportion of time spent at risk with habituation to the predator cues (Rodriguez-Prieto et al., 2009). We show that the risk allocation hypothesis accurately predicts the evolutionary response to constant predation risk for some types of behavior, but that prey evolved under variable predation risk only partially support the hypothesis.

3.3 Materials and Methods

In their model, Lima and Bednekoff (1999*b*) focused on two main measures of risk: the ratio between the attack rate of predators in high and low risk situations (α_H/α_L) and the proportion of time spent in high risk situations (ρ). Though both have effects on the predictions of the model, we focused on the the proportion of time spent at high risk because the populations we investigaed were evolved either at constant predation risk or with a single temporally unpredictable bout of risk. Because of this, the attack ratio was not meaningful for these populations as it was numerically undefined.

3.3.1 Episodic populations

A detailed description of the base population and the experimental evolution protocol for the episodic predation populations can be found in Chapter 2, but will be outlined briefly here. To generate the base population, a synthetic outbred population was created using an advanced intercross of 100 inbred lines collected from the wild in North Carolina and Maine (Goering et al., 2009; Reed et al., 2010). Following the intercross, the population was maintained at a large size, between 500-1000 flies, and allowed to mate randomly. After several generations of random mating, the synthetic outbred was then used to generate four populations that were assigned to two replicates. Each replicate consisted of a predator population and a predator free control population. The generational cycle of replicate 2 was offset by a few days due practical limitations, but was otherwise treated identically.

Predator populations were exposed to 1st instar nymphs of the Chinese mantis (*Tenodera aridifolia sinensis*) for one 24-hour bout of predation each generation after which all suriving flies were collected and allowed to lay eggs for the next generation. Mortality ranged between 10% and 80% with an average mortality of 40% per generation. Control populations were treated identically except that they were placed in arenas lacking mantids during the 24-hour predation period. Flies were typically aged 3-8 days old before undergoing predation. For clarity in distinguishing between continuous and episodic populations in this publication, these populations will be referred to as eCon and ePred. The episodic populations used in this experiment had undergone 91-93 generations of experimental evoluition in the variable predatation (ePredR1 and ePredR2) and predator free (eConR1 and eConR2) environments.

3.3.2 Continuous populations

A second outbred population, called FVW, was derived from wild caught flies collected at a winery in Southwest Michigan. The FVW population was used to generate 8 separate populations. Four populations were designated as controls and experienced no predation. The remaining four populations were exposed to continuous predation by 1st instar nymphs of *T. a. sinsensis*. Predator cages

were kept stocked with 30-40 mantids each (depending on seasonal availability). Both control and predation flies spent their entire lives in the individual cages.

To initiate the populations, approximately 1500 FVW flies were placed in 32.5x32.5x32.5 cm Bugdorm-43030 cages made out of polyester mesh with a single 200 mL culture bottle of food containing live yeast. Every four days a new bottle was added until there were a total of five bottles in each cage. From that point on, the oldest bottle was discarded every time an additional bottle was added leaving five bottles in each cage at any given time. After the 25 day rotation in the cage, the food inside the discarded bottle was completely consumed and all pupae within had eclosed. The cages were kept in a climate control room at approximately 24°C and 40% humidity and were sprayed with water daily to aid in humidification. Population sizes were not explicitly controlled in any way, but were generally smaller in the predation cages due to consumption by the mantids. Unlike the episodic populations where replicate and control populations were paired, predation and control replicates for the continuous populaitons were maintained independently of one another. These populations will be referred to as cCon and cPred.

In this study we measured only two of the continuous predation and control populations (cConR1, cConR2, cPredR4, and cPredR5). This was done not only to maintain balance with the two episodic replicates, but also because measuring all 8 populations proved unfeasable given contraints due to extensive preparation time and the duration of the assays. At the time the assays were conducted, the populations had been maintained for approximately two years. An exact estimate of the number of generations was unknown due to generational overlap among flies in the cages.

3.3.3 Risk assay

Flies from each population were collected after eclosion and aged 3-12 days before being assayed. Behavioral observations took place in 17.5x17.5x17.5 cm Bugdorm-41515 polyester mesh cages. Assay cages were washed each day with 70% ethanol and rinsed with distilled water. High risk cages were then stocked with 10 mantids each 18 hours before assays were performed to fast the mantids and allow their smell to build up inside the cages. Low risk cages remained empty and

were kept away from any mantids.

Assays were conducted over the course of 27 days between the end of April and the beginning of July, 2013 beginning at approximately 10 AM and concluding at approximately 2 PM. Observations were completely blocked so that each day all populations were assayed in high risk and low risk conditions. The order of the observations was randomized each day to control for potential effects of the time of day. Each assay consisted of 50 total flies (25 male and 25 female) that were introduced concurrently to each cage. Using scan sampling, we recorded every occurrance of the behaviors described in the following section by all of the flies in each cage. Each behavior was recorded as a discrete event using JWATCHER V1.0 (Blumstein et al., 2006). Behavioral recording began immediately after flies were added into the assay cage and continued for 10 minutes.

3.3.4 Behavioral observations

Courtship and aggression in *D. melanogaster* consist of suites of individually recognizable, stereotyped behaviors. These behaviors were chosen because they mediate important aspects of fitness in fly populations, and they were commonly observed in the presence of the predators in both episodic and continuous populations. Both sets of behaviors required investments of energy, time, and distract attention that might otherwise be spent on anti-predator activities. In addition, strong male biased mortality during the evolutionary process in the episodic populations suggested that these behaviors, primarily performed by males, would be likely targets of selection, and flies performing courtship activities have been observed being attacked and captured by mantids on numerous occasions.

For courtship, we recorded each time a male fly performed the following behaviors (*sensu* Lasbleiz et al., 2006):

(1) approach: orienting and walking toward a famale fly

(2) attempted copulation: curling of abdomen under thorax in attempt to contact genetalia with the female to initiate mating

(3) chasing: pursuing a female moving away from the male during courtship

(4) circling: moving in an arc around the female while maintaining orientation toward the female

(5) singing: extension and vibration of one wing blade at 90° from the body

For aggression, we recorded each time a fly of either sex performed the following behaviors (*sensu* Chen et al., 2002):

(1) chasing: running after another fly attempting to move away from it during an antagonistic interaction

(2) lunging: rearing up on hind legs and bringing the body and forelegs down on another fly (either male or female)

(3) wing threat: quickly raising both wings at 45° toward another fly (either male or female)

Though specific measures of vigilance have not been identified in *D. melanogaster*, we also recorded the following behaviors that flies in our populations perform in response to the presence of the mantids:

(1) abdominal lifting: rhythmic, upward undulation of the abdobem in the direction of a predator

(2) flying away: initiation of flight in response to the movement of a predator before a strike could take place

(3) running away: ambulatory locomotion in response to the movement of a predator before a strike could take place

(4) stopping: cessation of movement in close proximity to a predator

Finally, as a proxy for overall activity level, we recorded the number of voluntary flights initited during the observation period that were not in direct response to actions by the predators. We used

this measure because there wasn't an obvious way of measuring the rate of locomotion and attempting to track it prevented careful observation of the other behaviors in question. This measure did not completely capture total activity, but it was the best approximation possible based on our observations. All behavioral observations were performed and recorded by M. DeNieu.

3.3.5 Statistical analysis

For the analysis of each behavioral group (courtship, agggression, and anti-predator), we performed a principal components analysis of the individual behaviors (Table B.1). In each case, all individual behaviors loaded positively on the 1st principal component and were of similar magnitudes. Though only PC1 for courtship explained a majority of the variation, we focused solely on PC1 because it represented overall behavioral investment, and this was the primary focus of the risk allocation hypothesis. Successive PC's described differences in the performance of the individual behaviors (Table B.1). Though it is an interesting avenue for further study that, for example, a major axis of variation distinguished between aggression directed toward males and that directed toward females (see Table B.1, aggression: PC2), this distinction was not vital to this investigation. Furthermore, only PC1 was able to distinguish among the populations in an informative way for each of the behavioral groups, suggesting that, if variation in risk was driving the evolution of behavior, it does so through overall performance of all of these behaviors.

To estimate the reaction norms for courtship behavior, we used MCMCGLMM V2.21 in R V3.1.0 (R Core Team, 2012) to fit a mixed-effect, Markov chain Monte Carlo (MCMC), with the date each assay was performed as a random effect. We fit the fixed effects of risk regime (continuous or episodic), selection regime (predation or control), risk intensity (high or low), and their interactions as covariates. We also used mixed effect model with the random and fixed effects described above to estimate the reaction norms for aggression and overall activity. Because the anti-predator behaviors we measured we defined in regard to their interaction with the mantids, they could only be recorded in the high risk situation. As a result, we fit a mixed-effect, Markov chain Monte Carlo with date as a random effect, but only estimated fixed effects for risk regime,

selection regime, and their interaction.

All models were run with uninformative priors for two hundred thousand iterations resulting in an effective sampling rate of approximately ten thousand for each fixed effect and random effects after thinning and the burn-in period was removed. For all estimates reported, we used the median plus or minus 95% quantiles of the posterior distribution. Though there were some differences between replicates, their inclusion did not improve model support by deviance information criterion or change the important patterns of the results or conclusions. We focused on the overall differences between populations that were exposed to episodic predation risk and continuous predation risk for clarity.

3.4 Results

3.4.1 No evidence of differences among control (no predator) populations reared under the continuous or episodic selection regimes

A significant complication with this study was that the base populations for the episodic and continuous risk regimes differed with respect to the populations of origin and the maintenance of the populations during experimental evolution. Though this was unavoidable, we believe that we have strong evidence to suggest that it did not affect our conclusions. In the results that follow, we found no significant differences between the continuous and episodic control populations, suggesting that the naive behavioral patterns did not differ despite their differences in origin. Furthermore, we see no substantial differences in the correlation between aggression and courtship behaviors for any of the populations (Figure B.1). Thus, we do not have evidence that differences in evolutionary history had confounding effects on the experimental treatments.

3.4.2 Continuous and episodic predation populations lose plasticity for courtship behavior

To assess the effect of variability in predation risk on the evolution of behavioral plasticity, we observed the performance of aggression and courtship behaviors in populations evolved under

episodic and continuous predation risk. We found that the control populations significantly reduced their courtship behavior when at high predation risk (high risk = -1.27 ± 0.63 courtship activity, $p < 0.0001$; Figure B.2A). The reduction in courtship behavior in the episodic control populations was slightly less than in the continuous populations, but this difference was non-significant ($p \simeq 0.18$).

The risk allocation hypothesis predicts that prey experiencing relatively constant risk should not adjust their behavior between high and low risk situations. This means that in the continuous predation populations, we should observe a decrease in the reaction norm for courtship as compared to the controls. As predicted, the continuous predation populations showed a reduction in plasticity as compared to the controls (continuous x predation x high risk = 0.99 ± 0.9 courtship activity, $p \simeq 0.03$, Figure B.2B). Conversely, prey experiencing variable risk should reduce activity during high risk and increase it during low risk situations. This means that in the episodic populations, we should observe an increase in the reaction norm between high risk and low risk situations for courtship in the predator evolved populations as compared to the controls. However instead of increasing plasticity, the episodic predation populations showed an even greater reduction in plasticity than the continuous predation populations. Though not significantly different from the continuous populations ($p \simeq 0.93$), the loss of plasticity in these populations is counter to the predictions of the risk allocation hypothesis.

3.4.3 Predation populations do not reduce plasticity for aggression

For aggression, the continous control populations again showed a reduction in behavioral activity when at high risk (high risk = -1.01 ± 0.46 aggressive activity, $p < 0.0001$; Figure B.2C). The episodic control populations showed a slight, but non-significant ($p \simeq 0.30$), reduction in plasticity relative to the continuous controls. Though we predicted that the continuous predation populations should decrease plasticty and the episodic predation populations should increase plasticity, we instead found that aggression was reduced at high risk for both risk regimes, with only a slight, non-significant reduction in plasticity from the control populations ($p \simeq 0.41$; Figure B.2D).

3.4.4 Patterns of behavior for courtship and aggression do not represent changes in activity level

In addition, we wanted to determine if the evolved patterns for aggression and courtship differed from overall activity level. Preliminary observations suggested that the total number of non-predator related flights was a good, though not perfect, proxy for general activity level. Unlike the other behaviors measured, we found that flights were primarily determined by the selection regime, and that activity increased at high risk (high risk = 11.73 ± 6.94, $p \simeq 0.001$; Figure B.3). Continuous predation populations significantly reduced the number of flights at both risk levels (continuous x predation = -14.19 ± 7.0, $p < 0.0001$). Episodic predation populations showed even lower activity levels (episodic x predation = -12.29 ± 9.71, $p \simeq 0.013$).

3.4.5 Episodic predation populations increase anti-predator behaviors as predicted

In addition to courtship and aggression, we recorded potential anti-predator behaviors that were performed during exposure to the mantids. The "paradox of risk assessment" predicts that prey that experience variable risk should increase their investment into anti-predator activities when at high risk, and prey that experience constant risk should show lower levels of investment. This means that in the predator evolved populations, we should observe a large increase in anti-predator activities in the episodic populations as compared to the controls and a decrease in the continuous populations as compared to their respective controls.

All control populations performed anti-predator behaviors at a similar rate (-0.16 ± 0.41 and -0.13 ± 0.46 anti-predator activity for continous and episodic populations, respectively, $p \simeq 0.53$; Figure B.4). Consistent with the predictions of the risk allocation hypothesis, constant predation risk in the continuous populations did not select for an increase in anti-predator behavior (continuous x predation = 0.03 ± 0.44 anti-predator activity, $p \simeq 0.87$). Also consistent with predictions, variable predation risk in the episodic populations did select for an increase in anti-predator behavior (episodic x predation = 0.88 ± 0.65 anti-predator activity, $p \simeq 0.007$)

3.5 Discussion

As with several previous studies, our results provide mixed support for risk allocation. The predation populations exhibited a similar level of plasticity as the controls for aggression (Figure B.2C & D). All populations, regardless of their predation history, increased overall activity, as measured by the number of flights initiated, at high risk (Figure B.3). The predation populations showed reduced activity as compared to the controls but at both risk levels. We did find that the changes in courtship observed in the continous predation populations supported the risk allocation hypothesis, but those in the episodic predation populations did not (Figure B.2A & B). In fact, the level of courtship in the episodic predation populations was greater at high risk than any of the other populations. Differences in the base populations of the continuous and episodic risk regimes could affect these results. However, because we found no significant differences between the controls populations of the two risk regimes, it is unlikely that this played a significant role.

Studies have shown that foraging animals are able to ameliorate some of the dangers they face by modifying their behavior, affecting the predictions of risk allocation. Though not as quickly as vigilant individuals, dark-eyed juncos were shown to detect the approach of a simulated predator even while foraging, presumably by engaging in less effective head down vigilance between feeding pecks (Lima and Bednekoff, 1999*a*). Resident elk experienced with variation in predator abundance were also shown to increase the multitasking of vigilance and chewing in response to risk, potentially reducing the effect of greater vigilance on the rate of foraging. (Robinson and Merrill, 2013). Finally, gerbils were shown to alter feeding rates in accordance with the phases of the moon so that feeding was most efficient when the moon was dark and there was less need for vigilance (Kotler et al., 2010).

Similarly, we found that the riskiness of the particular behaviors had a strong effect on their response to selection. Courtship behaviors require a significant amount of attention to the opposite sex for both males and females, and often result in long bouts of locomotion that bring them in contact with predators. Nearly 50% of courtship behaviors involved locomotion including pursuit, approaching, and circling (Figure B.5A), and we observed several courtship events that led directly

to capture of one of flies by the mantids (often the female). Consistent with this, we observed strong effects of selection by predators on courtship behavior in both episodic and continuous populations, even though they only partially supported the hypothesis. By contrast, over 80% of all aggressive behaviors performed were wing threats, which are nearly instantaneous and unlikely to detract from vigilance or other anti-predator behaviors (Figure B.5B). It is then, perhaps, unsurprising that the predator-evolved populations did not differ significantly from the control populations as these behaviors are unlikely to significantly increase overall risk.

However, when taken in aggregate with the anti-predator behaviors, which do support the risk allocation hypothesis, our data might suggest an alternative explanation. Aggressive behaviors do not appear to pose a significant risk, and have not been greatly modified by selection. Courtship behavior does appear to pose a significant risk, and makes up a majority of the total behaviors performed by the populations (Figure B.6). As a result, we have seen a reduction in overall courtship in both predation populations. The loss of plasticity for courtship in both predation populations suggests that it does not benefit individuals to vary their behavior in response to changes in risk. However, increases in anti-predator behavior in the episodic predation populations supports the idea that it only benefits to invest in anti-predator behaviors when risk varies.

The original formulation of the risk allocation hypothesis framed vigilance and activity as mutually exclusive scenarios, yet we know that animals possess a wide range of behavioral strategies for reducing predation risk that extend beyond vigilance (Lima and Dill, 1990). These behaviors are performed in addition to and not instead of other behaviors. Our data suggest that, if we want to understand how prey animals respond to predation, we need to not only understand the variation in risk that prey experience, but we must also measure a more complete behavioral repertoire as some types of behavior constitute greater risk than others.

Furthermore, most previous studies have made a distinction between learned responses to risk, in which organisms can make flexible changes to their behavior, and genetic effects that are a result of selection for fixed responses to particular contexts. It is not clear that such a distinction is useful or warranted because the extent to which the two responses are independent or even different is

not known. Animals can and do adjust their behavior, but this is undoubtedly within the context of their evolutionary history. Here we showed that populations do evolve strategies consistent with risk allocation in response to selection by predators. A greater understanding of the risk dynamics under which populations are evolving can only strengthen our understanding of their behavior, and when taken into account in further study, may also strengthen support for risk allocation.

3.6 Acknowledgments

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CHAPTER 4

SIZE DOESN'T MATTER, IT'S HOW YOU USE IT: THE ASSOCIATION BETWEEN ALLOMETRY AND ANTI-PREDATOR BEHAVIOR

4.1 Abstract

The divergence of wing size and shape in populations of *Drosophila melanogaster* that have been evolved in response to selection by nymphs of the Chinese mantis (*Tenodera aridifolia sinensis*) led us to hypothesize that there are multiple strategies for avoiding predation. We assayed the escape response, avoidance behavior, and several important morphological traits of these experimentally evolved populations to begin to understand the evolutionary changes that have occurred. Our results show that the relative scaling between body parts may be an important determinant of whether or not the flies successfully escaped during a predator attack and not overall size itself. Populations that primarily used flight to escape attacks were successful when wing loading and leg loading were low even though there were differences in absolute size. The population that showed increased use of locomotion to escape attacks was also successful even with higher wing and leg loadings. However, the combination of high wing loading and low leg loading was unsuccessful in escaping predation. These results suggest that the relative scaling between body parts may have been an important factor determining whether a particular strategy was successful, yet the primary targets of selection remain unclear.

4.2 Introduction

Behaviors are unique among phenotypic traits for their remarkable flexibility and the rapidity with which they can be altered in response to shifts in the environment. As a result, it has long been recognized that selection on behavior can be an important driver of evolution within and among populations experiencing rapid environmental change (Wcislo, 1989; West-Eberhard, 1989). This

process, originally described by Baldwin (1896) and now known as the Baldwin effect (Simpson, 1953), is a multistep process in which environmentally induced phenotypes could become fixed in a population through the process of natural selection.

First, organisms in a population encounter a new environment to which they are poorly adapted. Some individuals within this population exhibit plasticity for a phenotype, in our case a behavioral trait, that preadapts them to survive in the new environment. As a result, these individuals attain higher fitness. Over many generations, natural selection acts on available genetic variation to increase the frequency of alleles that increase or improve performance of the trait directly, and on genetic variation in other associated traits through the process of correlational selection (Brodie III, 1992; Sinervo and Svensson, 2002).

The exposure of prey to new predators is one situation that may be likely to drive evolutionary change in this manner because of the many ways in which prey organisms can behaviorally respond to predation risk (Lima and Dill, 1990). Prey can attempt to avoid encountering predators either temporally or spatially, and if they do encounter a predator, optimal escape strategies may depend on the type of predator and the context for the interaction (Cooper and Frederick, 2010). Furthermore, the tight correlation between behavior and morphology suggests that selection on escape behavior may also impose correlated selection on the size and scaling of morphological characters.

Many studies on populations exposed to new predators have showed morphological changes associated with increased locomotor efficiency when escape behavior was important for survival. Damselfly larvae in lakes where fish were the primary predators attempted to avoid predator encounters by moving slowly and infrequently, and did not attempt to actively escape predators. However, when introduced to fishless lakes, damselflies switched to a swimming strategy and possessed larger lamellae and wider abdomens that resulted in greater swimming ability in order to escape the ambush attacks of dragonfly larvae that were the dominant predators (McPeek, 1995). Similarly, many fish and amphibian species have been shown to evolve torpedo-like bodies with narrow heads and broad tails in response to the introduction of predators (Dayton et al., 2005;

Langerhans et al., 2004; Langerhans and Makowicz, 2009; O'Steen et al., 2002). This body plan has been shown to improve the fast start behavior used to escape predators by increasing propulsion (Webb, 1984). Anolis lizards introduced to experimental islands with predators evolved longer legs in males and larger body size in females, both of which were correlated with increased running speed (Losos, 1990; Losos et al., 2004). In avian species, allometric relationships between the body and wings affected aerial maneuverability and have been shown to be associated with particular escape strategies (Hedenström and Rosén, 2001).

In Chapter 2, we presented evidence that replicate populations of *Drosophila melanogaster* evolved in response to selection by a novel mantid predator had diverged in wing size over the course of evolution despite being subjected to the same selection procedure. We hypothesized that selection on anti-predator behaviors had led to correlated selection on aspects of morphology suited to the different strategies. Since those experiments were performed, we have observed a striking behavioral difference between our predation populations during normal fly maintenance. Flies from predation replicate 2 would not initiate flight in response to mechanical disturbance. Behavior like this is extremely rare for flies in general, and especially so for these populations.

We hypothesized that the unique behavior we observed might have been due to changes in escape behavior. In order to investigate these changes, we first sought to document this behavior by observing the response to a simulated attack in the evolved populitions. We next asked if these changes in escape behavior might be due to an increased avoidance response in this population. Finally we examined the association between morphology and anti-predator behavior in these populations in order to determine if the complex patterns of morphological evolution we previously observed were due to association with escape or avoidance behavior. We show that though all populations avoided predators in the same manner, there were significant differences in the escape behavior and that certain allometric relationships were associated with the successful escape strategies.

4.3 Materials and Methods

4.3.1 Experimental populations

Flies for all assays described below were derived from populations that had been experimentally evolved in the presence of predators and from their respective predator free controls. This experimental process was described in detail in Chapter 2, but the important details will be summarized here. We generated a synthetic outbred population using an advanced intercross of 100 inbred lines that were collected from the wild in Maine and North Carolina (Goering et al., 2009; Reed et al., 2010). Afterward, flies from the synthetic outbred population were allowed to mate randomly and were maintained at a population size of approximately 500-1000 individuals for several generations. We used this population to generate four individual populations that were then split into two replicates. One population from each replicate was assigned to the predator selection regime and the second to the predator-free selection regime, which served as a control. We offset the generational cycle of replicate 2 by several days for logistical purposes, but it was treated identically otherwise.

We subjected the predator populations to viability selection by 1st instar nymphs of the Chinese mantis (*Tenodera aridifolia sinensis*) during one, 24 hour bout each generation. All surviving flies were then collected and placed in a cage with food bottles, allowing them to lay eggs for the next generation. Control populations underwent the same procedure, except that the arenas in which they were placed in during the 24 hour period did not contain predators. Flies were typically aged 3-8 days before undergoing selection. In the following sections, populations from the first replicate will be referred to as PredR1 and ConR1, and populations from the second replicate will be referred to as PredR2 and ConR2. All data were recorded by M. DeNieu.

4.3.2 Escape response assay

Escape response, in Drosophila, is a reflexive response that differs in speed and neurological control from voluntary flight (Card and Dickinson, 2008*a*,*b*). To investigate the unique behavior we

observed and to help determine the strategies used to escape predator attacks, we tested the response of the evolved populations to a simulated attack because it should be representative of their response to a real predatory attack. To do this, we placed individual flies aged 3-7 days at generation 71 of experimental evolution inside a narrow plastic vial with a small dab of fly food on the side-wall to attract the fly to a consistent location and provide humidity between trials. We then covered the opening with cotton (Figure C.2). Flies were given 30 minutes to acclimate to the arena before testing and in between repeated measures to ensure that they had settled before each test. When testing, the blunt end of a paintbrush was slowly placed inside the edge of the vial, so as not to disturb the fly, and then quickly pushed toward it to elicit a response. Each individual within a block was tested in succession and allowed an additional 30 minutes between repeated trials.

The escape response assays were performed over four days from February 14-17, 2012. Two blocks of assays were performed each day with 3 males and females from each of the four evolved populations (PredR1, ConR1, PredR2, ConR2) in each block. The first block on each day was performed between 10:30 AM and 2:00 PM. The second block was performed between 2:30 PM and 6:00 PM. Individuals in each block were tested 4 times, except for block 2 on day 1 which was tested 3 times and block 1 on day 3 which was tested 5 times. In a few trials, a fly escaped from the vial during the course of the trials. In these cases, we used the remaaing trials to measure the escape response on the new fly. All trials were recorded with a JVC Everio GZ-MS230 video camcorder.

Flies performed one of two escape responses. If the fly jumped away from the approaching paintbrush and initiated flight it was said to have used a "flying" escape strategy. If instead the fly remained on the ground and used locomotion to move out of the way of the printbrush it was said to have used a "dodging" strategy. In addition, we recorded whether the fly was on the bottom of the vial, along the side-walls, or on the cotton in order to control for the effect of location on escape response.

4.3.3 Avoidance assay

To test whether changes in escape beahvior we associated with changes in predator avoidance, we observed the locomotory behavior of individual flies in isolation and then in the presence of a heterospecific insect. We used nymphs of the Chinese mantis (*Tenodera aridifolia sinensis*) with which the predation populations were evolved as a heterospecific predator that was known to the evolved populations. We used nymphs of the house cricket (*Acheta domesticus*) as a heterospecific control because they are non-predatory, unfamiliar to the flies, and we could easily obtain individuals that matched the size of the mantids. We used the control insect to determine whether the behavioral response to the mantis was due to recognition as a predator or simply due to the introduction of a foreign insect. We chose the crickets because in our initial trials, the crickets were a close match to the activity level of the mantids.

Locomotory behavior was observed in arenas consisting of the bottom half of an overturned 100 mm Petri dish placed onto one quarter of a 33x30 cm glass pane (Figure C.1). Petri dishes were held in place using hardened dabs of hot glue as chocks. A small hole was made in the side of each dish in order to create an opening through which flies could be aspirated. Predator and control insects were added to the arena through this opening as well. During assays, the opening was covered with a fresh piece of cotton.

Underneath the glass plane, we placed a sheet of paper with 1 mm squares so that the approximate travel distance and the distance from the fly to the predator could be measured. Dividers was placed between arenas to prevent individuals in one arena from seeing those in the others. A total of three arrays were constructed so that 12 total flies could be assayed at any given time. Each set of four arenas was illuminated by an overhead LED lamp and recorded with a JVC Everio S MS230 video camcorder.

Avoidance assays were performed over the course of three days on March 30 and April 2-3, 2012. Each day four blocks of assays were performed, beginning at approximately 10:30 AM and proceeding until approximately 2:00 PM. A single block consisted of 12 individual assays performed concurrently with the order randomized each day. Males and females from each of

the four evolved populations (PredR1, ConR1, PredR2, and ConR2) were assayed with a predator in every block. Half the males and females from each population were assayed with a control insect in the first block. The remaining half were assayed in the second block. This process was randomized and repeated for the third and fourth blocks. In total, 32 males and females from each population were assayed with a predator each day, and 16 males and females from each population were assayed with the control insect.

Flies from each predation and control population at generation 74 of experimental evolution were collected after eclosion and aged 3-7 days in mixed sex groups before being assayed. They were then separated into vials containing only males or only females 24 hours before each assay in order to speed the process of aspirating flies into the arenas. At the beginning of each block, all three camcorders were set to record and individual flies were added into each arena in succession. Each fly was left alone in the chamber for at least 5 minutes, after which the predator and control insects were added to their respective arenas. After an additional 5 minutes the camcorders were stopped and all insects were removed from each arena. The glass pane was wiped with 20% ethanol per lab protocol, rinsed with distilled water. At the end of each day, all used Petri dishes were cleaned with 20% ethanol and rinsed with distilled water for use on the next day.

From the videos, we recorded the number of 1 mm squares the fly moved across at five second intervals when the fly was alone and after the addition of the predator or control insect. A thirty second acclimation period was given after the initial addition of the fly to the arena to allow it to settle before recording began. Recording during the predator phase began as soon as the mantis or cricket entered the arena. In addition, we recorded the coordinate distance (X, Y) between the fly and the head of the predator or control insect every 5 seconds and used it to calculate the straight line distance between them.

4.3.4 Survival assay

In Chapter 3, we measured the behavioral response of the evolved populations from generations 91-93 to the presence of predators over the course of 27 days between 10 AM and 2 PM each day.

Populations were measured in the late morning and early afternoon because this coincided with the the time period in which the predation populations were exposed to predators during experimental evolution. During the 10 minute assay, 25 male and 25 female flies aged 3-12 days old were exposed to predation by 10 mantid nymphs in 17.5x17.5x17.5 cm Bugdorm-41515 polyester mesh cages. Because the mantids were not separated from the flies in any way and were not prevented from attacking them, predation attempts did occur. We recorded each time a predator captured a fly in the 10 minute period. These data were used as our proxy for survival ability for each evolved population. In particular, this measure of survival should have most closely reflectd successful ability to escape predators because the flies and mantids had so little time to interact.

4.3.5 Morphological characters

We finally wished to determine if there was an association between the behavioral responses to predators and morphology. To do this we measured thorax length (as a proxy for body size), wing size, and lengths of all three legs on individual flies. To first control for parental effects we reared each population in common garden conditions at low denisty for 2 consecutive generations beginning at generation 99 of experimental evolution.To keep flies at low density we allowed flies to lay eggs on grape juice agar for several hours, after which eggs were picked from the surface and placed into vials at low density (30 eggs per vial). After the eggs were placed into their respective vials, they were placed in an incubator at 24°C and 60% humidity to develop. After eclosion, flies were aged 2 days to allow for the cuticle to fully scleratize and were then placed into 70% ethanol for dissection.

Flies were dissected one at a time in 70% ethanol. Images of the thorax were taken on the right side of the fly. Wings and legs were always dissected from the right side, unless they showed evidence of damage, and laid flat before imaging. All images were captured at 40X magnification with a Leica DFC400 camera mounted on a Leica M125 microscope using the LEICA APPLICATION SUITE software for image capture. Images were saved in greyscale as TIFF files.

To calculate wing size, we used a modified protocol from Pitchers et al. (2013) for the WING-

MACHINE software (Houle et al., 2003). The program TPSDIG2 V2.17 (Rohlf, 2010) was first used to manually record the coordinates of two starting landmarks, and then WINGMACHINE was used to fit nine B-splines to the veins and margins of the wings in the images. We extracted 14 landmark and 34 semi-landmark positions, and performed Procrustes superimposition (Zelditch et al., 2012). After superimposition, we used CPR V0.2 (Marquez, 2010) to allow the positions of semi-landmarks to slide along each segment of the wing margin and veins in order to minimize the Procrustes distance. We then extracted centroid size for use as a measure for overall wing size (Zelditch et al., 2012). The thorax was measured as the straight line distance between the anterior tip of the prescutum and the posterior tip of the scuttellum. Legs were measured along the center of the limb from the proximal tip of the femur at the articulation with the trochanter to the tip of the tarsal segment. Thorax length and leg lengths were measured manually using IMAGEJ V1.48E (Abramoff et al., 2004). In addition to the measures for size we also calculated the wing loading (body size/wing size) and leg loadings (body size/leg size) for each individual.

4.3.6 Statistical analysis

4.3.6.1 Escape response

To determine the proportion of times dodging for each population, we fit a mixed-effect model to estimate the fixed effects of selection regime, replicate, the interaction between selection regime and replicate, and location (bottom, side, or cotton). As a random effect we estimated the effect of individual accounting for each trial.

4.3.6.2 Avoidance

To determine average speed of flies when alone, we fit a mixed effect markov chain Monte Carlo with the distance traveled per 5 second interval as the dependent variable. We fit the fixed effects of selection regime, replicate, and the interaction between replicate and selection regime, and time elapsed in seconds as covariates. We also fit the random effect of each individual (each

fly measured) accounting for time allowing for the estimation of heterogeneous variances and the covariance between them. We fit an identical model to estimate average speed with the heterospefic insect present and the average distance between the fly and the heterospecific insect, except that in both cases we added the fixed effect of heterospecific type (mantis or cricket) and its interactions with selection regime and replicate.

4.3.6.3 Survival

In Chapter 3 we performed experiments in which we recorded the behavior of flies in cages with mantids for 10 minutes. During these trials we recorded the number of flies captured and eaten in each trial. In order to estimate the average number of flies eaten in this 10 minute period for each population, we fit a mixed effect, markov chain Monte Carlo with the date each assay was performed as a random effect and the fixed effects of selection regime (predation or control), replicate (R1 or R2), and their interaction.

4.3.6.4 Morphology

To estimate wing size, thorax length, leg lengths, wing loading and leg loadings for each population we fit separate linear models with the main effects of selection regime, replicate, sex, and the interaction between selection regime and replicate.

4.3.7 Statistical estimation

All analyses were performed in R v3.1.1 (R Core Team, 2012). Mixed effect models were fit using Markov chain Monte Carlo methods using MCMCglmm v2.21 with default settings for uninformative priors. We ran each model for two hundred thousand iterations resulting in an effective sampling rate of approximately ten thousand for each fixed effect and random effects after thinning and the burn-in period was removed. For all parameter estimates from mixed effects models reported in the text, we used the posterior mean plus or minus 95% credible intervals. Figures show the median of the posterior distribution for each effect plus or minus the 2.5% and

97.5% quantiles as error bars. Parameter estimates reported from linear models are means plus or minus standard errors. Support for mixed effects models was evaluated by deviance information criterion (DIC). Support for linear models was evaluated using Bayesian information criterion (BIC). In all cases we used the model with the lowest DIC or BIC that included the parameters of interest.

4.4 Results

4.4.1 PredR2 perform significantly more dodges

Consistent with our previous observations, ConR1, ConR2, and PredR1 primarily initiated flight in response to the simulated attack, and only performed dodges at low proportions. However, PredR2 showed a significant increase in the proportion of dodging behavior compared to the other populations (predation x R2 = 0.17 ± 0.11, $p \simeq 0.007$, Figure C.3).

4.4.2 All populations reduce locomotion in the presence of predators

We hypothesized that the reduction in flight in PredR2 might be due to an increased ability to avoid the predators.To test this hypothesis, we then performed a second experiment where we observed the locomotion of flies in the absence and presence of the mantids and contrasted this with their activity in the presence of a control insect with which they should be unfamiliar. We found that ConR2 had a higher average speed than ConR1, PredR1, and PredR2 with the predator absent (R2 = 5.62 ± 4.37 mm, $p \simeq 0.012$, Figure C.4). In addition, the presence of the cricket did not significantly change the average speed of any of the populations, though the control populations did show a slight, non-significant increase in speed as compared to the predation populations (Figure C.4). However, all populations reduced their locomotion in the presence of the mantis (predator = -13.7 ± 7.25 mm, $p < 0.0002$). The estimates of the reduction in locomotion were lower for PredR1 (-8.01 mm) and PredR2 (-10.11 mm) than for the controls, but these interactions were non-significant ($p \simeq 0.28$ and $p \simeq 0.49$ respectively). It is not clear whether this difference is simply a

result of the slightly positive increase in locomotion in the controls in the presence of the cricket, or reflects something about the evolved response of the predation populations. To shed additional light on this, we also measured the distance each fly kept from the predator or control insect. All populations maintained the same distance to the mantis as they did to the cricket (distance to heterospecific = -2.37 ± 7.84 mm, $p \simeq 0.56$, Figure C.6).

4.4.3 PredR1, PredR2, and ConR2 show increased survival

Our results for avoidance behavior suggested that there had not been selection to avoid predator encounters in PredR1 or PredR1. As a part of another experiment, we had measured survival over a 10 minute period with the mantids. Because of the short time period, the number of predator interactions was limited, suggesting that escape behavior was likely more important than during the full 24 hour assay. We found that the predation populations significantly reduced their capture rate as compared to ConR1 (selection regime = -0.74 ± 0.47, $p \simeq 0.003$). We also found that ConR2 had a significantly reduced capture rate (replicate = -0.85 ± 0.47, $p < 0.001$, Figure C.5).

4.4.4 Allometric relationships associated with survival not overall size

As we had previously seen, PredR1 and PredR2 showed increased survival ability. However, ConR2 also showed a similar ability to survive in this assay. We wanted to know how morphology related to these patterns. We found that differences in body size among the populations were primarily determined by replicate with both ConR2 and PredR2 being larger than ConR1 and PredR1 (R2 = 0.017 ± 0.006 mm, $p \simeq 0.003$, Figure C.7A). A similar pattern was found for wing size. ConR1 had the smallest wings, and ConR2 had the largest wings (R2 = 0.15 ± 0.023 mm, $p < 0.0001$, Figure C.7B). The wings of PredR2 were slightly smaller than its control (R2 x predation = -0.10 ± 0.033 mm, $p \simeq 0.002$). The wings of PredR1 were significantly larger than its control (predation = 0.06 ± 0.02 mm, $p \simeq 0.01$), but were smaller than both ConR2 and PredR2. Size differences among the four populations was different for the legs (Figure C.8). These results suggested that morphological loadings may be more important than absolute size.

Though we did not see differences in overall size, we also wanted to investigate the alometric relationships between body size, the wings and legs.We calculated wing loading (body size/wing size) and leg loading (body size/leg size) for each of the populations. We found that PredR1 and ConR2 had nearly identical wing loadings, which were lower than ConR1 (predation = -0.0018 ± 0.0009, $p \simeq 0.043$ and R2 = -0.0017 ± 0.0009, $p \simeq 0.045$). The wing loading for PredR2 was simialr in magnitude to ConR1 (predation x R2 = 0.0033 ± 0.0012, $p \simeq 0.008$). PredR1 and ConR2 had leg loadings that were similar in magnitude to ConR1, (predation = -0.006 ± 0.003, $p \simeq 0.06$ and replicate = -0.001 ± 0.003, $p \simeq 0.65$). However, the leg loading for PredR2 was much higher than the other populations (predation x R2 = 0.029 ± 0.005, $p < 0.0001$). The values reported above were calculated using leg 1 only as it is representative of the loadings for leg 2 and leg 3 since the size differential among populations was nearly identical for each leg (Figure C.8).

When we plotted the correlation between wing loading and leg loading, we saw that ConR2 and PredR1 cluster in the lower left quadrant, PredR2 is in the upper right quadrant, and ConR1 is in the upper left quadrant (Figure C.9). Populations in the lower left quadrant had low wing and leg loading, and primarily utilized quick flights in response to a simulated predator attack. The population in the upper left had low wing loading and high leg loading and also primarily utilized flight in response to the simulated predator. The population in the upper right had high wing and leg loadings, but utilized an increased dodging strategy to avoid the simulated predator strike. Successful escape by flight was only observed in PredR1 and ConR2 which had low wing loading and leg loading. Successful escape by dodging was only observed in PredR2, which had high wing and leg loadings.

4.5 Discussion

Biologists have long understood the importance of allometric scaling relationships to the study of development, anatomy, physiology, and evolution (Gould, 1966). Allometric relationships are equally important to the study of behavior because of the functional constraints imposed on the performance of behavior by the morphology of the organism (Dial et al., 2008). The goal of this

study was to investigate the relationship between morphology, behavior, and fitness in populations that had been experimentally evolved with predators in light of the unique dodging behavior that we observed. We measured overall body size, wing size, leg lengths, and calculated loadings for these traits. We also measured the ability of each population to avoid predators and escape simulated attacks. We found that both predator populations evolved increased survival ability (Figure C.5) yet appeared to have accomplished this using different escape strategies (Figure C.3). PredR1 primarily utilized a flying strategy and PredR2 utilized a mixed flying and dodging strategy. More unexpectedly, we found that ConR2 also exhibited survival ability equal to the predator populations despite having never been exposed to predation.

We not find that the increase in dodging in PredR2 led to greater avoidance behavior. None of the populations significantly changed their locomotion in the presence of the cricket as compared to their baseline movement, and all populations reduced their locomotion in the presence of the mantid (Figure C.4). This is consistent with the predicted response to an ambush predator (Wirsing et al., 2010) and may suggest that all populations retain the ancestral ability to recognize the mantids as a predatory threat. However, all populations maintained the same distance to mantis and the cricket (Figure C.6). The difference in locomotion between the mantids and crickets may have been due to the increased activity of the cricket, but given that all of the populations responded identically, we do not have good evidence that the avoidance behavior is a primary target of selection.

Our results do suggest that there may be a relationship between escape behavior, wing loading and leg loading (Figure C.9). Escape response flight in *Drosophila melanogaster* is a complex series of actions that differs from normal voluntary flight. Originally, it was thought that the initial thrust off the ground was produced solely by the extension of the second set of legs. However, it has recently been shown that wing extension is an important part of the preparation for escape flight (Hammond and O'Shea, 2007). When responding to an oncoming stimulus, the fly first adjusts its body position, begins extending its wings, and then the primary motor force is generated by the extention of the legs and a quick downstroke with the wings toward the body (Card and Dickinson,

2008*a*,*b*).

Consequently, reduction in wing and leg loading should increase takeoff velocity due to the increased thrust produced by the wings and legs (Berrigan, 1991). Therefore, despite the absolute difference in size between ConR2 and PredR1, both flies possessed morphology conducive to quick escape flight. Though ConR1 possessed equivalently low leg loading to ConR2 and PredR1, the relative increase in wing loading may have slowed its takeoff velocity enough to cause more frequent capture by the mantids. On the other hand, PredR2 appeared to have been able to ameliorate the slight increase in wing loading and substantial increase in leg loading by decreasing its reliance on escape flight to avoid capture.

We originally hypothesized that variation in anti-predator behaviors might have led to morphological divergence (Wcislo, 1989; West-Eberhard, 1989). Though we presented evidence of an association between escape response, wing loading, and leg loading, we do not have direct evidence to suggest that selection on the dodging and flying escape responses caused the observed changes in morphology because these experiments were performed on different sets of flies .

The major differences in body size and wing size was between replicates, suggesting that this was due to founder effects, drift, or inadvertent selection unrelated to predation, and not to selection on escape behavior. In one respect, because the control populations should be representative of all non-predatory selection acting on these populations, it seems likely that, if selection on escape response is driving the evolution of morphology, PredR1 should have evolved the dodging strategy, given that ConR2 appears to be preadapted to successfully escape predation. On the other hand, these inconsistencies may support the hypothesis that behavioral evolution is driving the changes in morphology because the patterns are so different from what we might predict.

Historical contingency has been shown to be extremely important in determining the outcome of evolutionary changes, particularly in the laboratory (Blount et al., 2008; Simões et al., 2008). Chance increases in successful behavioral strategies, escape flight in PredR1 and dodging in PredR2, may have led to indirect selection for allometric relationships that improved performance of these strategies. Alternatively, differences in allometric relationships due to founder effects

might have constrained the successful behavioral strategies available to each population during the course of evolution. Additional tests are required to directly show that the observed differences in escape behavior are responsible for differences in survival. In addition, tests need to be done using within population variation to untangle the association between morphology, escape behavior and survival. Though the results of this study are not conclusive, they present evidence that the association between morphology and behavior has been an important factor in the evolutionary outcome, highlighting the importance of understanding morphology in the context of behavior, and also the challenges of understanding the process of adaptive evolution even in controlled environments.

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APPENDICES

APPENDIX A

CHAPTER 2: TABLES AND FIGURES

	Model 1	Model 2	Model 3
(Intercept)	6.71	7.14	7.33
	(0.38)	(0.50)	(0.60)
Sex: M	-2.91^{***}	-2.94^{***}	-3.33^{***}
	(0.55)	(0.55)	(0.84)
Date: June 2008		-0.77	-1.03
		(0.59)	(0.82)
Date: March 2008		-0.57	-1.33
		(0.89)	(1.40)
Sex: M x Date: June 2008			0.53
			(1.20)
Sex: M x Date: March 2008			1.33
			(1.84)
R^2	0.42	0.45	0.46
Adj. R^2	0.41	0.40	0.38
Num. obs.	41	41	41
ΔAIC	0.0	3.1	8.1
ΔBIC	0.0	5.5	12.2
ΔDeviance	7.4	1.8	0.0
BIC Weights	0.937	0.061	0.002

$^{***}p < 0.01$, $^{**}p < 0.05$, $^{*}p < 0.1$

Table A.1 **Model selection for survival in the base population.** Survival ability in the base population measured as the number of surviving flies in each arena after 24 hours exposure with the predators. Table shows the output from the LM funtion in R for a set of models evaluated using Bayesian information criteria. Numbers in parenthesis are standard errors of the above estimates.

	Model 1	Model 2	Model 3	Model 4	Model 5
(Intercept)	10.86	10.97	11.41	10.99	11.32
	(0.40)	(0.45)	(0.49)	(0.50)	(0.60)
Selection Regime: Pred	3.12^{***}	3.11^{***}	3.11^{***}	3.07^{***}	3.26^{***}
	(0.40)	(0.41)	(0.40)	(0.57)	(0.71)
Generation: 32	4.83^{***}	4.83^{***}	4.00^{***}	4.83^{***}	4.24^{***}
	(0.90)	(0.90)	(0.99)	(0.91)	(1.08)
Eggcase: B	-1.61^{**}	-1.61^{**}	-1.62^{**}	-1.61^{**}	-1.60^{**}
	(0.80)	(0.80)	(0.79)	(0.80)	(0.80)
Eggcase: C	-4.73^{***}	-4.75^{***}	-4.85^{***}	-4.75^{***}	-4.85^{***}
	(1.34)	(1.35)	(1.34)	(1.35)	(1.34)
Eggcase: D	-4.00^{***}	-4.00^{***}	-4.00^{***}	-4.00^{***}	-4.00^{***}
	(0.90)	(0.90)	(0.89)	(0.90)	(0.90)
Eggcase: E	2.16^{*}	2.16^{*}	2.16^{*}	2.16^{*}	2.14^{*}
	(1.25)	(1.25)	(1.24)	(1.25)	(1.25)
Sex: M		−0.21	-1.06^{*}	−0.25	−1.17
		(0.40)	(0.58)	(0.58)	(0.73)
Sex: M x Generation			1.65^{**}		1.66^{**}
			(0.80)		(0.81)
Selection Regime: Pred x Sex				0.09	0.20
				(0.81)	(0.81)
Selection Regime: Pred x Generation					−0.47
					(0.81)
R^2	0.43	0.43	0.44	0.43	0.44
Adj. R^2	0.41	0.41	0.42	0.41	0.42
Num. obs.	210	210	210	210	210
ΔAIC	0.3	2.2	0.0	4.4	4.1
ΔBIC	0.0	5.1	6.0	10.4	16.3
ΔDeviance	41.2	39.0	3.4	38.9	0.0
BIC weights	0.882	0.07	0.043	0.005	< 0.001

$^{***}p < 0.01$, $^{**}p < 0.05$, $^{*}p < 0.1$

Table A.2 **Model selection for survival in the evolved populations.** Survival ability in the evolved populations measured as the number of surviving flies in each arena after 24 hours exposure with the predators. Table shows the output from the LM funtion in R for a set of models evaluated using Bayesian information criteria. Numbers in parenthesis are standard errors of the above estimates.

	Model 1	Model 2	Model 3
(Intercept)	0.18	0.29	0.32
	(0.12)	(0.18)	(0.20)
Wing size	-0.32^{**}	-0.41^{**}	-0.46^{**}
	(0.12)	(0.17)	(0.20)
Sex: M		-0.28	-0.21
		(0.35)	(0.38)
Wing size x Sex			0.17
			(0.39)
Num. obs.	294	294	294
ΔAIC	0.0	1.4	3.3
ΔBIC	0.0	5.1	10.5
ΔDeviance	0.83	0.19	0.0
BIC weights	0.921	0.074	0.005

$^{***}p < 0.01$, $^{**}p < 0.05$, $^{*}p < 0.1$

Table A.3 **Model selection for selection on wing size in the Base population.** The output from the logistic regression of wing size onto survival in the base population for a set of models evaluated using Bayesian information criteria. Numbers in parenthesis are standard errors of the above estimates. The logistic regression models were used to evaluate statistical significance of the estimated selection differentials. The values reported in the manuscript were taken from identical linear regression models (output not shown)

	Model 1	Model 2	Model 3	Model 4	Model 5
(Intercept)	0.50	0.53	0.64	0.86	0.79
	(0.11)	(0.11)	(0.12)	(0.15)	(0.16)
Wing size	-0.42^{***}	-0.35^{***}	-0.40^{***}	-0.62^{***}	-0.55^{***}
	(0.09)	(0.09)	(0.09)	(0.13)	(0.14)
Selection Regime: Pred	0.55^{***}	0.31^{***}	0.16	0.16	0.16
	(0.07)	(0.09)	(0.11)	(0.11)	(0.11)
Replicate: R2	-0.27^{***}	-0.60^{***}	-0.57^{***}	-0.57^{***}	-0.57^{***}
	(0.10)	(0.13)	(0.13)	(0.13)	(0.13)
Sex: M	-0.45^{***}	-0.29^{*}	-0.34^{*}	-0.73^{***}	-0.76^{***}
	(0.17)	(0.18)	(0.18)	(0.23)	(0.23)
Generation: G32	-0.48^{***}	-0.43^{***}	-0.63^{***}	-1.02^{***}	-1.01^{***}
	(0.11)	(0.11)	(0.14)	(0.20)	(0.20)
Wing size x Selection Regime	0.19^{***}	0.20^{***}	0.22^{***}	0.24^{***}	0.23^{***}
	(0.07)	(0.07)	(0.07)	(0.07)	(0.07)
Replicate: R2 x Generation	0.96^{***}	1.02^{***}	1.03^{***}	1.09^{***}	1.09^{***}
	(0.14)	(0.14)	(0.14)	(0.15)	(0.15)
Sex M x Generation	0.69^{***}	0.64^{***}	0.64^{***}	1.42^{***}	1.38^{***}
	(0.14)	(0.14)	(0.14)	(0.33)	(0.33)
Selection Regime: Pred x Replicate		0.60^{***}	0.58^{***}	0.53^{***}	0.52^{***}
		(0.15)	(0.15)	(0.15)	(0.15)
Selection Regime: Pred x Generation			0.35^{**}	0.32^{**}	0.33^{**}
			(0.14)	(0.14)	(0.14)
Wing size x Generation				0.44^{***}	0.44^{***}
				(0.17)	(0.17)
Wing size x Sex					-0.19
					(0.17)
Num. obs.	3932	3932	3932	3932	3932
ΔAIC	23.6	8.9	4.8	0.0	0.7
ΔBIC	8.4	0.0	2.2	3.6	10.6
ΔDeviance	30.95	14.25	8.12	1.3	0.0
BIC weights	0.001	0.658	0.224	0.108	> 0.001

$^{***}p < 0.01$, $^{**}p < 0.05$, $^{*}p < 0.1$

Table A.4 **Model selection for selection on wing size in the evolved populations.** The output from the logistic regression of wing size onto survival in the evolved populations for a set of models evaluated using Bayesian information criteria. Numbers in parenthesis are standard errors of the above estimates.The logistic regression models were used to evaluate statistical significance of the estimated selection differentials. The values reported in the manuscript were taken from identical linear regression models (output not shown)

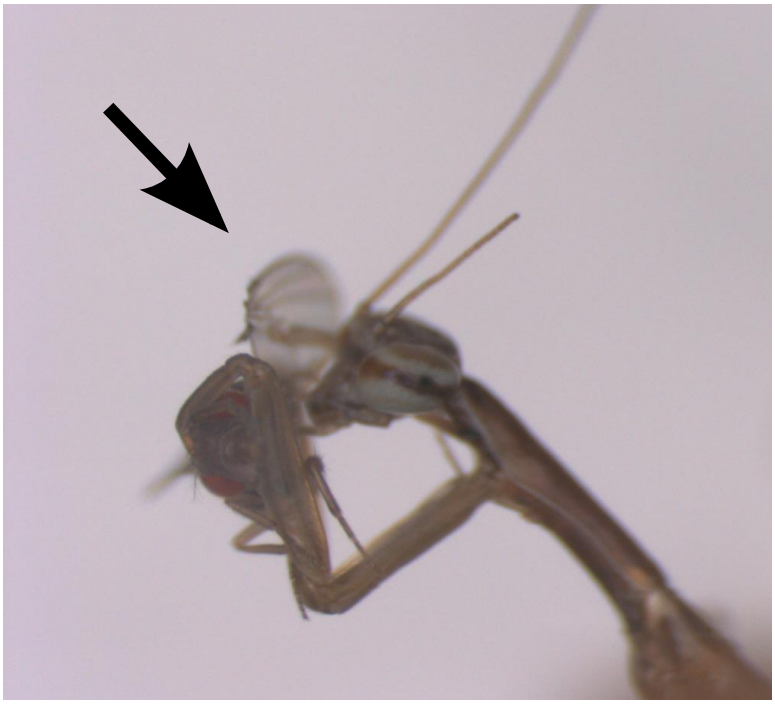

Figure A.1 **Mantis consuming a fly.** 1st instar nymph of the Chinese mantid (*Tenodera aridifolia sinensis*) consuming a fruit fly. Note the wing about to drop off.

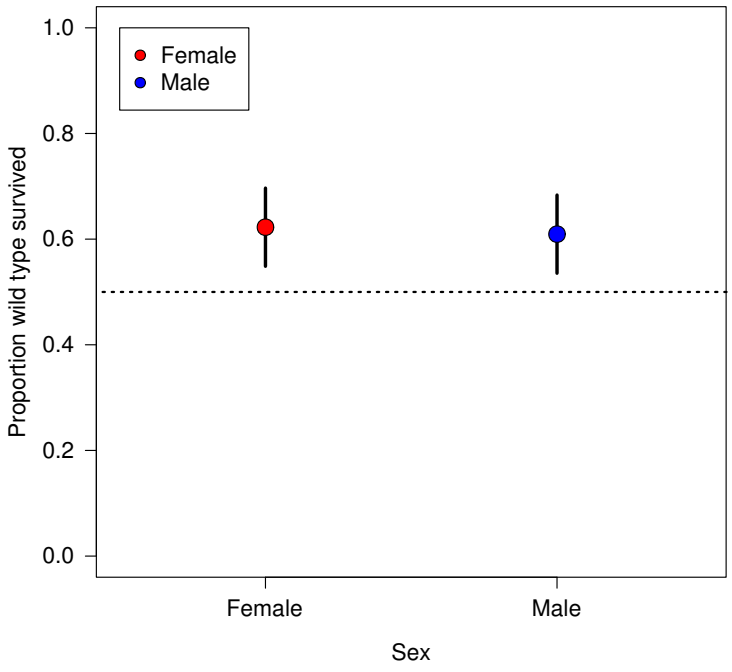

Figure A.2 **Impact of wing loss on survival.** Proportion of wild-type flies surviving in each arena. Error bars are 95% confidence intervals.

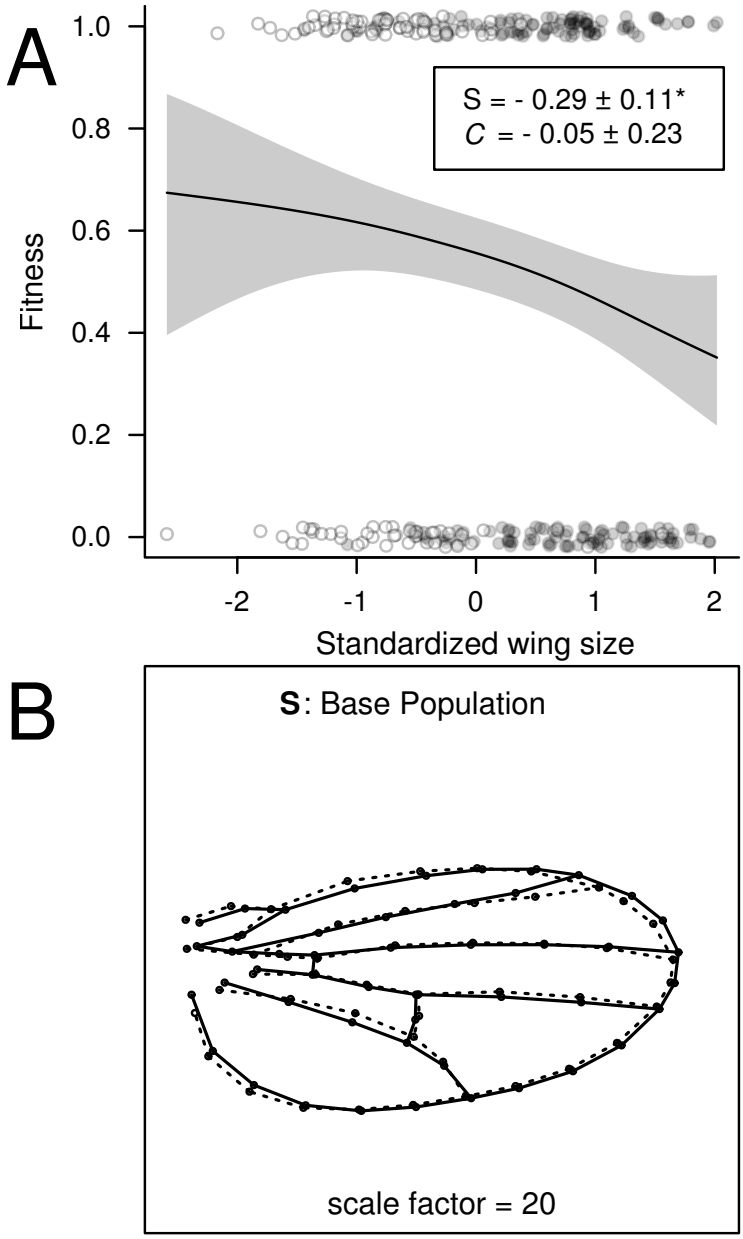

Figure A.3 **Evidence for selection on wing size and shape in the base population.** (**A**) The selective function for size estimated by fitting cubic splines (*sensu* Schluter, 1988) along with estimates for linear and quadratic selection. Stars denote significance from logistic regression, but estimates are derived from a linear regression of size on relative fitness. Points above the function are individuals that survived. Points below the line were captured and eaten. Dark filled dots are females and white filled dots are males. Error bands are 95% confidence intervals. (**B**) Visualization of the selection differential for shape **S** as measured in the base population. Points indicate landmarks and semi-landmarks. The shapes represent the mean shape plus 10x **S** (solid line) and minus 10x **S** (dotted line).

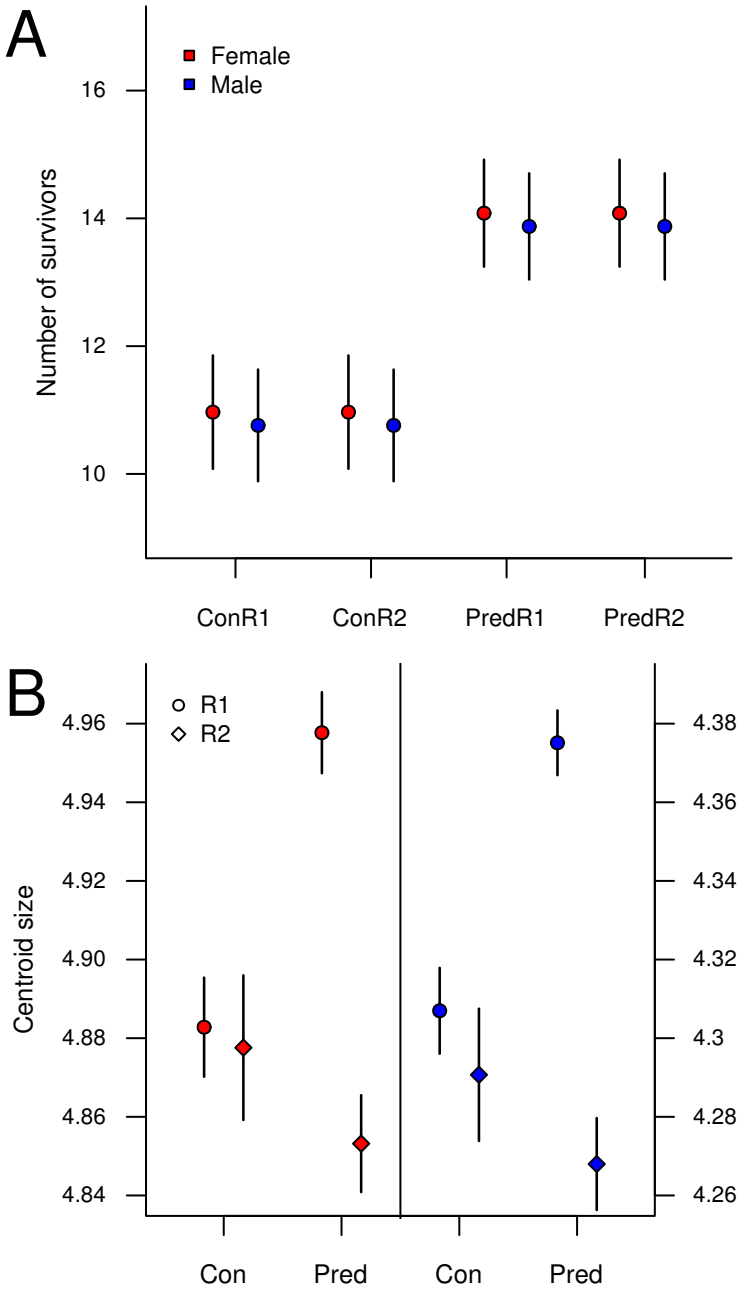

Figure A.4 **Predator populations show evolution of viability and wing size.** (**A**) Mean number of survivors in each predation arena after 30 generations of experimental evolution. (**B**) Differences in wing size of the evolved populations after 30 generations of experimental evolution. Points in red are females corresponding to the left axis, and points in blue are males corresponding to the right axis. Replicate 1 is shown in circles, and replicate 2 in diamonds. Errors are 95% confidence intervals.

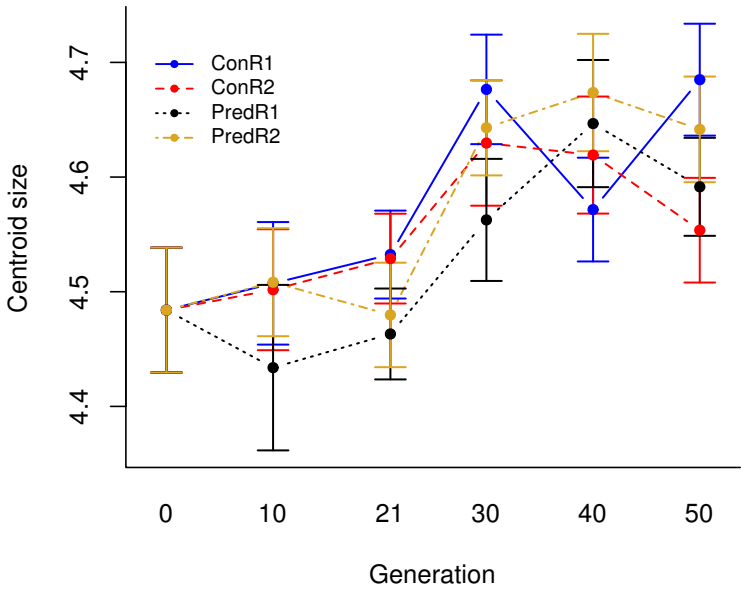

Figure A.5 **Evolved populations show increased size over 30 geenerations.** Mean wing size in each evolved population after 50 generations of experimental evolution. Wing sizes were measured on individuals every 10 generations that were stored during the experimental evolutionary process. Individuals from generation 21 were used because we did not have an archived population for generation 20. Errors are 95% confidence intervals.

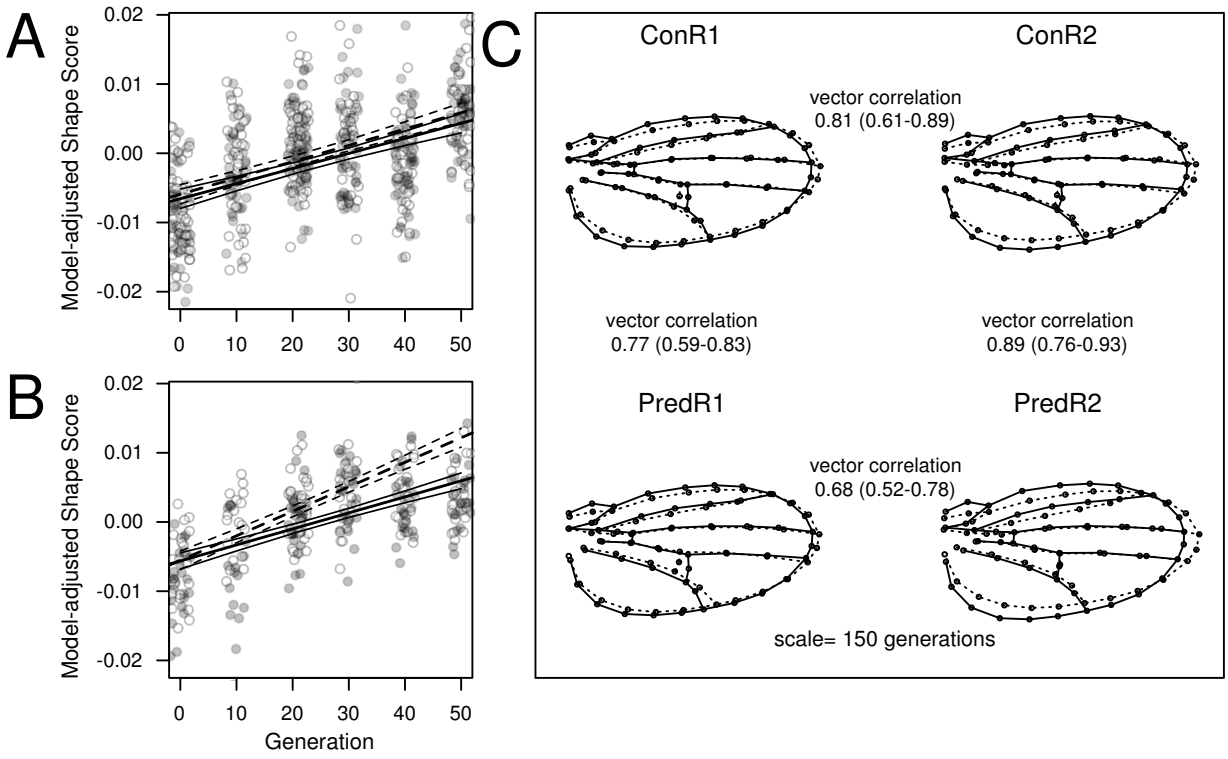

Figure A.6 **Magnitude and direction of shape change in the evolved populations** Shape score by generation for (**A**) control and (**B**) predation selection regimes. Model adjusted shape score for generation (*sensu* Drake and Klingenberg, 2008; see methods) is plotted against generation number, with white filled points for males and dark filled points for females. Solid regression lines and 95% confidence intervals are for replicate 1, and dashed lines and 95% confidence intervals are for replicate 2. (**C**) Visualization of the directions of the evolution of wing shape in the 4 experimental evolution populations. The shapes represent the mean plus (solid line) and minus (dotted line) the modelled vector of evolutionary change in each case, scaled to 50 generations in magnitude. The points represent landmarks and semi-landmarks. Vector correlations between these modelled directions of shape evolutions (and their 95% credible intervals) are printed between the pairs of populations to which they relate.

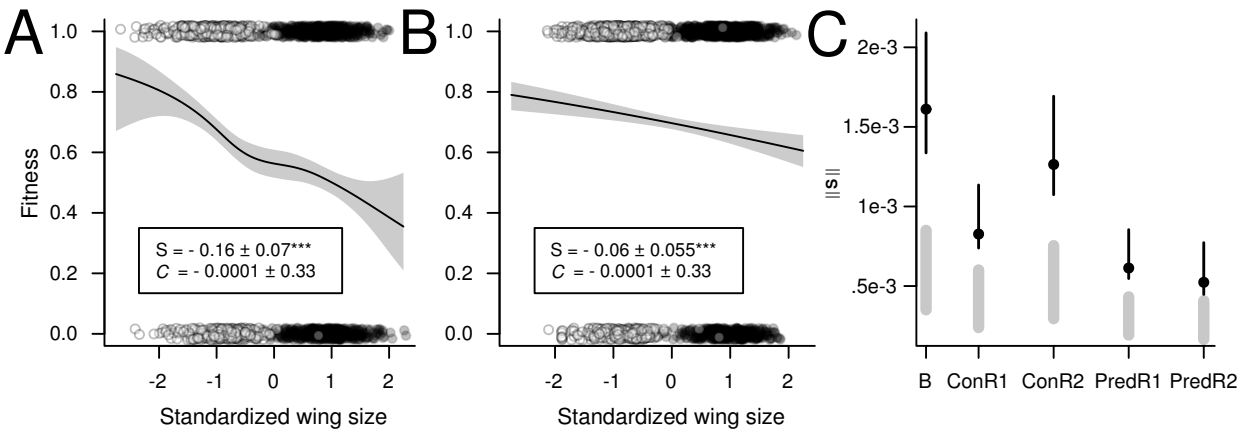

Figure A.7 **Patterns of selection on wing size and shape in the evolved populations** The selective function for size estimated by fitting cubic splines (*sensu* Schluter, 1988) with replicates pooled for the (**A**) control (**B**) and predation populations along with estimates for linear and quadratic selection. Points above the function are individuals that survived. Points below the line were captured and eaten. Dark filled dots are females and white filled dots are males. Error bands are 95% confidence intervals. (**C**) Magnitude of the selection differential **S** for shape as measured in the base (b), control (ConR1 and ConR2), and predation (PredR1 and PredR2) populations. Black points and lines are estimates and bootstrapped 95% confidence interval. The grey lines are the 95% confidence intervals from permutation of the same data; they represent the null hypothesis that the magnitude of **S** is random relative to survival.

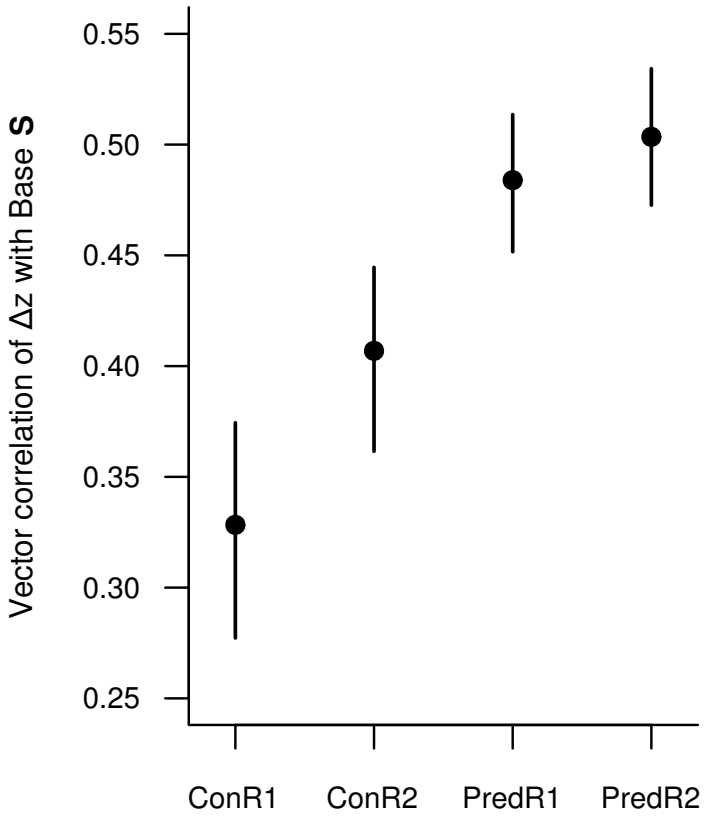

Figure A.8 **Correlation between selection and shape change in the evolved populations.** Vector correlations between **S** for wing shape estimated in the base population, and the direction of shape change during experimental evolution. The response vector was estimated within each population. Points are vector correlation estimates, and lines represent 95% bootstrapped confidence intervals.

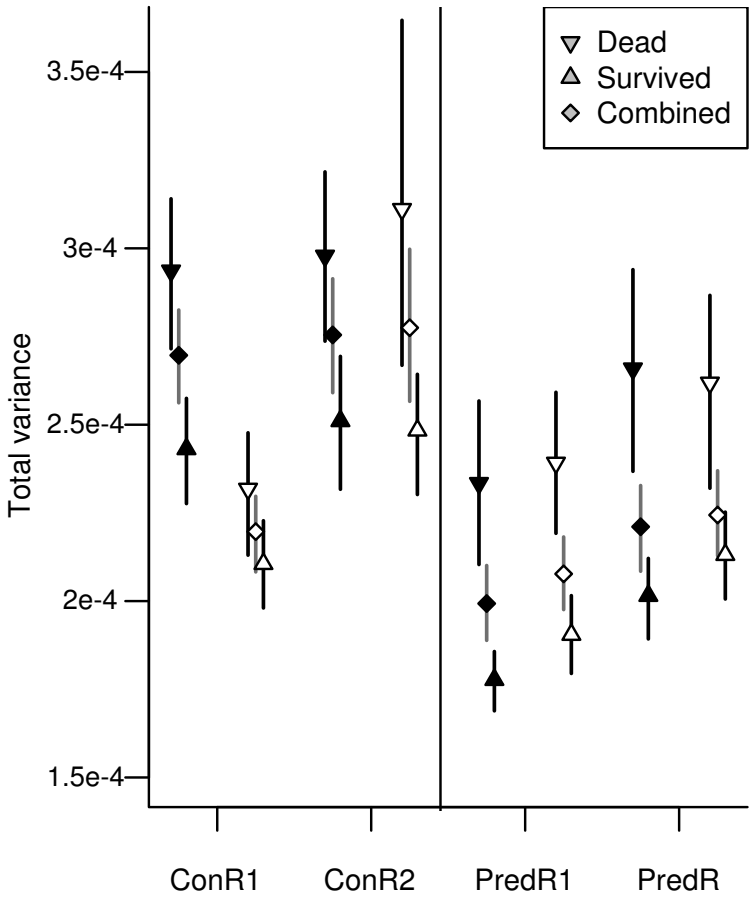

Figure A.9 **Patterns of variation for shape in response to predation in evolved populations.** Estimates of variance for shape calculated as the trace of the covariance matrix for female (filled points) and male (open points) flies from the evolved populations. Estimates of total variance (diamonds) are calculated with dead and surviving flies combined. Error bars are 95% bootstrapped confidence intervals.

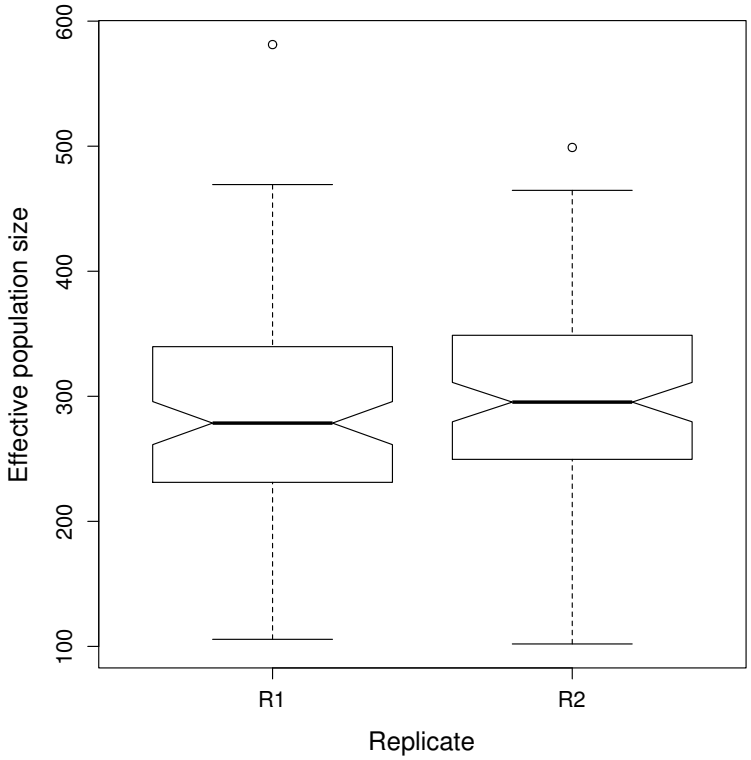

Figure A.10 **Variation in effective population size over 30 generations of experimental evolution.** Because the population size of the control populations was matched to that of the predation populations, control and predation effective population sizes were identical for each replicate..

APPENDIX B

CHAPTER 3: TABLES AND FIGURES

Courtship					
	PC1	PC2	PC3	PC4	PC5
Circling	0.44	0.48	-0.43	0.52	-0.34
Att. copulation	0.47	-0.03	0.72	0.42	0.28
Singing	0.48	-0.14	-0.47	-0.22	0.69
Approach	0.47	0.32	0.25	-0.71	-0.32
Chasing	0.36	-0.80	-0.11	0.04	-0.47
% Variance	0.73	0.16	0.05	0.03	0.03

Aggression (target)					
	PC1	PC2	PC3	PC4	PC5
Wing threat (female)	0.26	-0.64	0.04	-0.70	0.16
Wing threat (male)	0.58	0.24	-0.10	-0.18	-0.75
Lunging (female)	0.27	-0.63	-0.36	0.63	-0.10
Lunging (male)	0.53	0.03	0.76	0.28	0.25
Chasing	0.49	0.37	-0.53	-0.05	0.58
% Variance	0.36	0.26	0.14	0.13	0.11

Anti-predator				
	PC1	PC2	PC3	PC4
Stopping	0.40	-0.76	0.51	0.01
Run away	0.59	0.09	-0.32	-0.73
Fly away	0.57	-0.02	-0.48	0.67
Ab. lift	0.41	0.64	0.63	0.13
% Variance	0.50	0.20	0.20	0.10

Table B.1 **Results of principal components analysis of courtship, aggression, and anti-predator behavior.** Loading of behavioral variables onto principal components and the amount of variation explained each for each behavioral group.

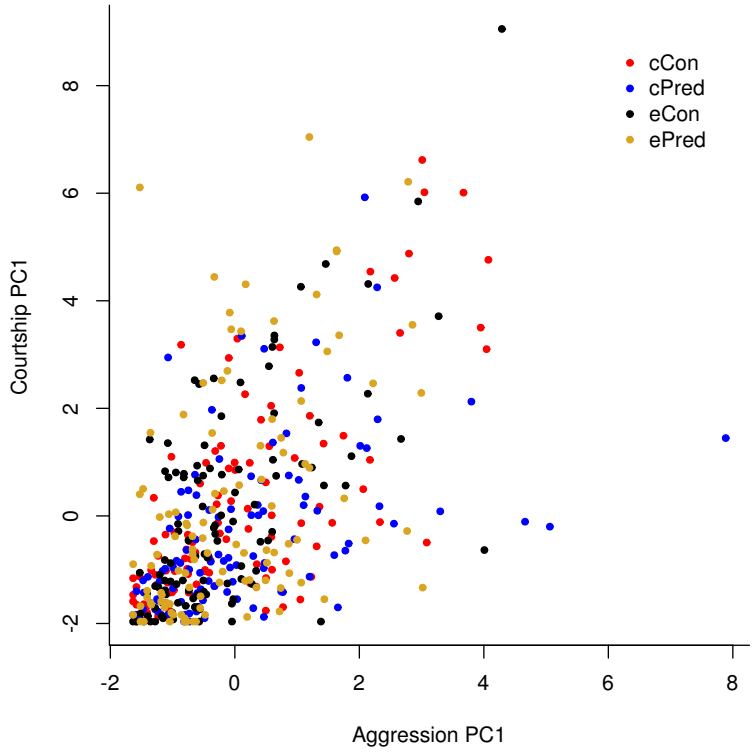

Figure B.1 **Correlation between PC1 for courtship and aggression.** Points represent values for individual cages for continuous control (red), continuous predation (blue), episodic control (black), and episodic predation (goldenrod) populations.

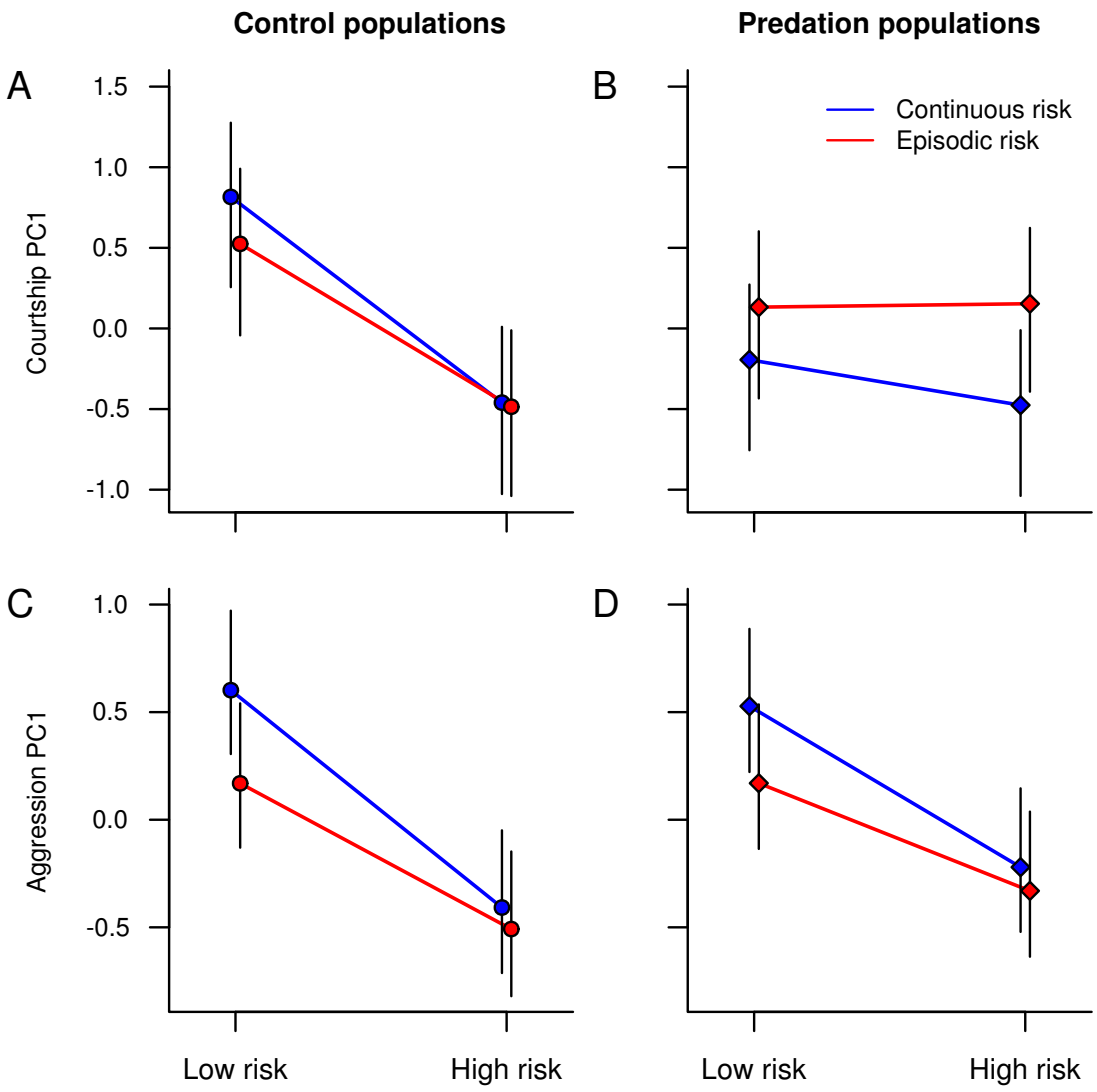

Figure B.2 **Reaction norms for courtship and aggression behaviors between high and low risk situations.** Points represent the median of the posterior estimates of PC1 for the continous (blue) and episodic (red) populations for both control (circles) and predator (diamonds) selection regimes. Control populations showed significant reduction of (**A**) courtship and (**C**) aggression when predators were present. Predation populations showed a loss of plasticity for (**B**) courtship, but only a slight (n.s.) reduction in plasticity for (**D**) aggression. Error bars are 95% confidence intervals.

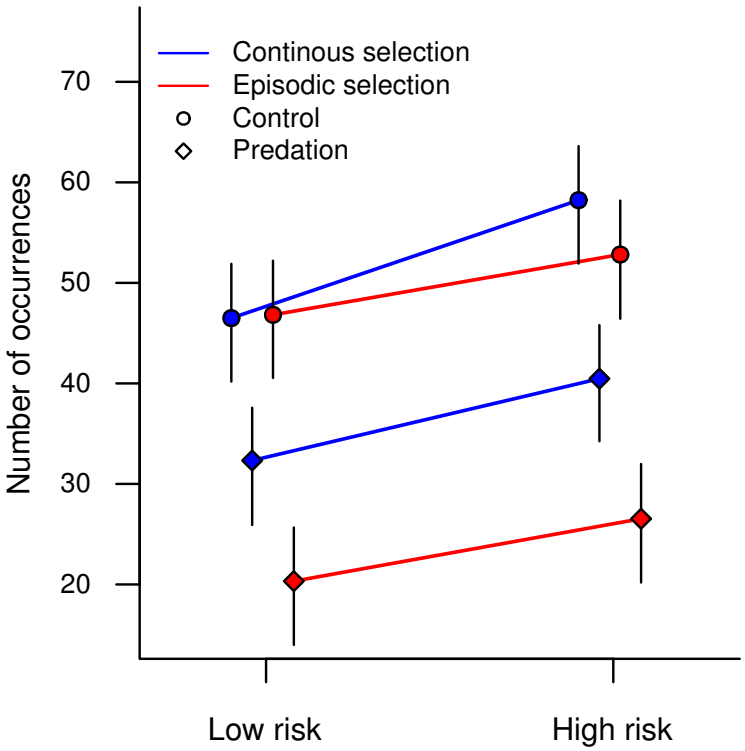

Figure B.3 **Reaction norms for activity level.** Points are the average number of non-predator related flights initiated for the continous (blue) and episodic (red) populations for both control (circles) and predator (diamonds) selection regimes. All populations showed a slight increase in activity when predators were present, but the primary differences are population specific. Error bars are 95% confidence intervals.

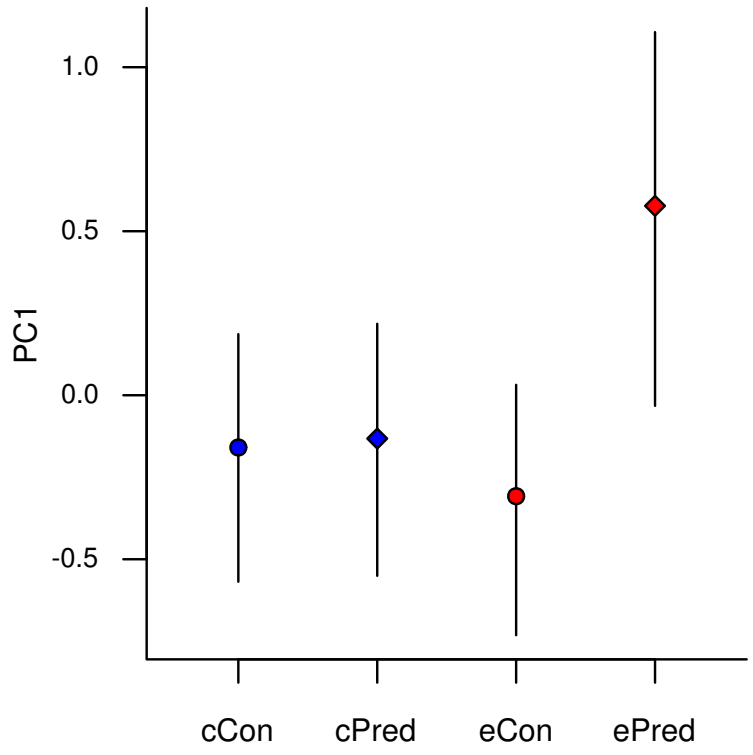

Figure B.4 **Total anti-predator behaviors performed in the presence of the predators.** Anti-predator behaviors include abdominal lifting, flying away, running away, and stopping performed in response to action by the mantids. Error bars are 95% confidence intervals.

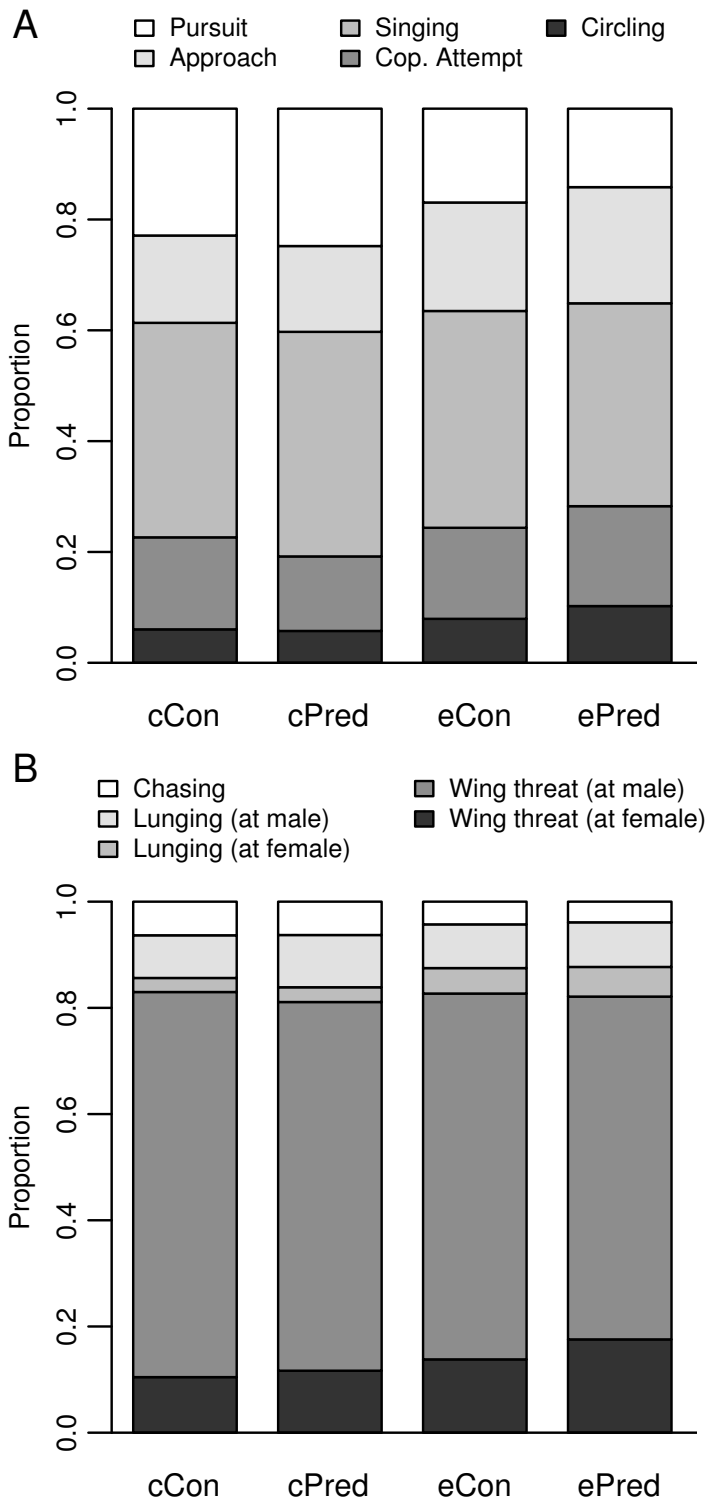

Figure B.5 **Contribution of individual behaviors to total courtship and aggression.** Shaded regions represent the total number of times each behavior was performed as a proportion of total (**A**) courtship and (**B**) aggression.

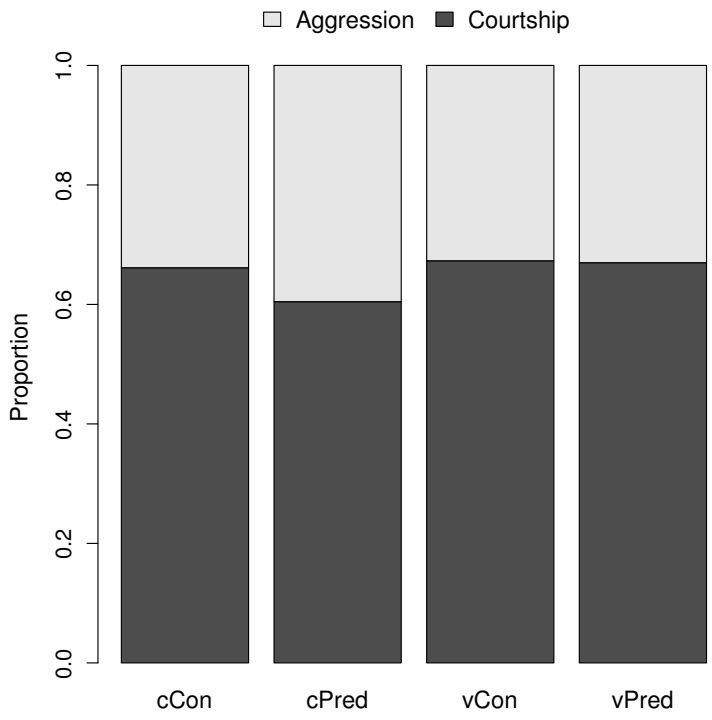

Figure B.6 **Relative performance of aggressive and courtship behaviors.** Shaded regions represent the total number of times each type of behavior was performed as a proportion of total aggressive and courtship behaviors.

APPENDIX C

CHAPTER 4: TABLES AND FIGURES

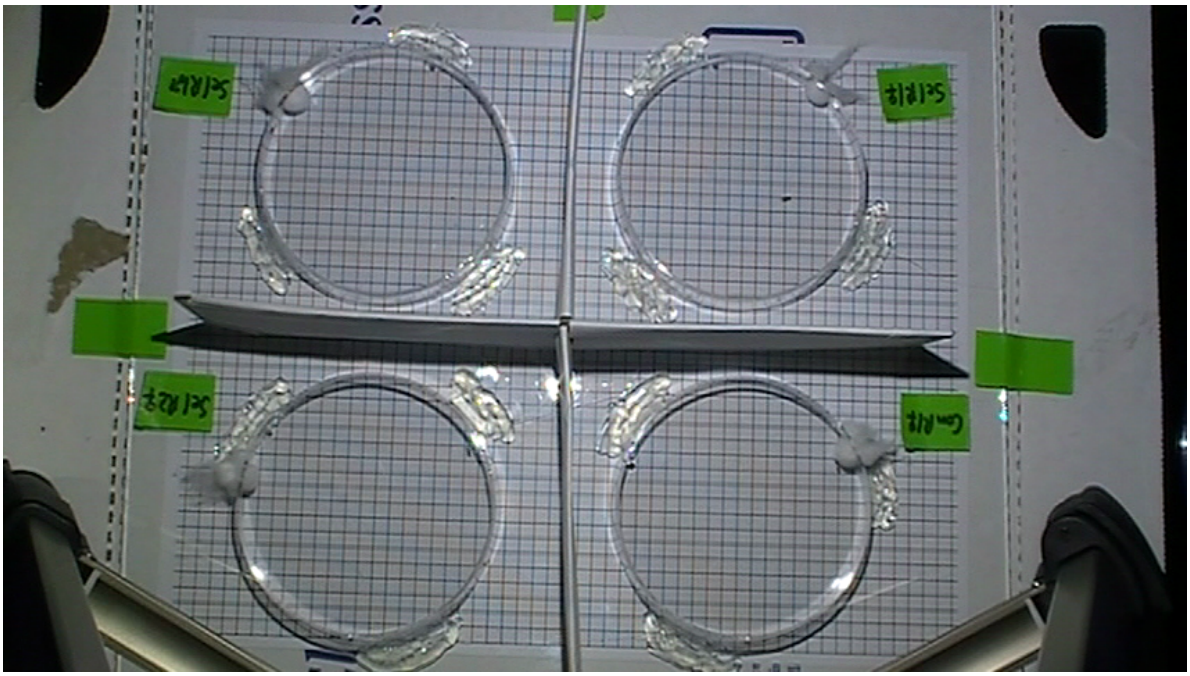

Figure C.1 **Experimental arenas for testing the predator avoidance behavior of *Drosophila melanogaster*.** The above image shows one of three identical stations used to measure avoidance behavior. Each fly was video recorded in an overturned Petri dish for 5 minutes on its own to determine baseline locomotory behavior and then for 10 minutes after the addition of a predator or control insect. Every 5 seconds the number of 5 mm squares crossed was recorded. In the presence of the predator, the X and Y distance from the fly to the head of the predator was also recorded.

Figure C.2 **Experimental arenas for testing the escape response of *Drosophila melanogaster*.** The response of each fly was video recorded to determine wether the fly evaded the simulated attack by jumping to initiate flight (flying) or by using ambulatory locomotion to move out of the way (dodging). During each trial, it was recorded wether the fly was on the cotton, the side-wall, or the bottom of the vial when the simulated attack was performed.

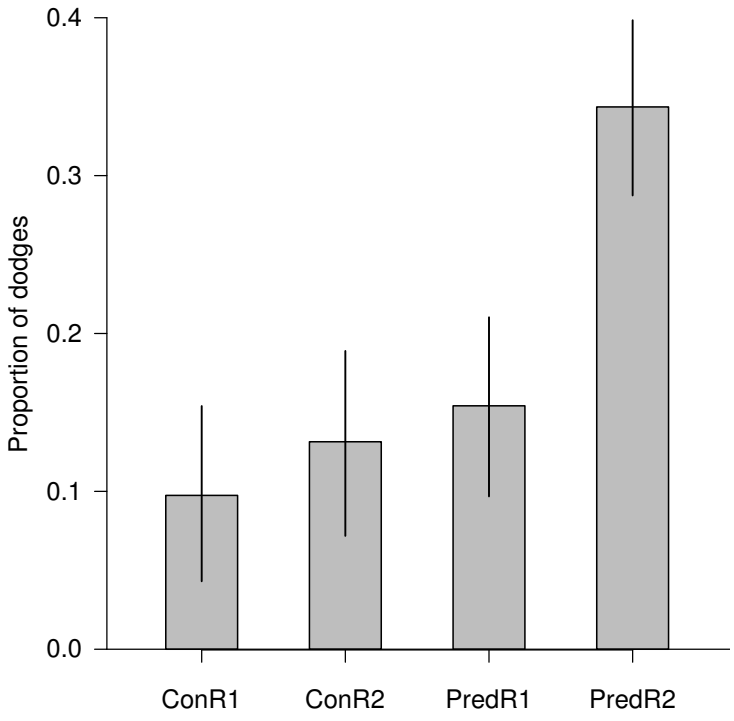

Figure C.3 **Escape responses of predation and control populations** Proportion of times dodging in response to a simulated predator attack. Error bars are 95% confidence intervals.

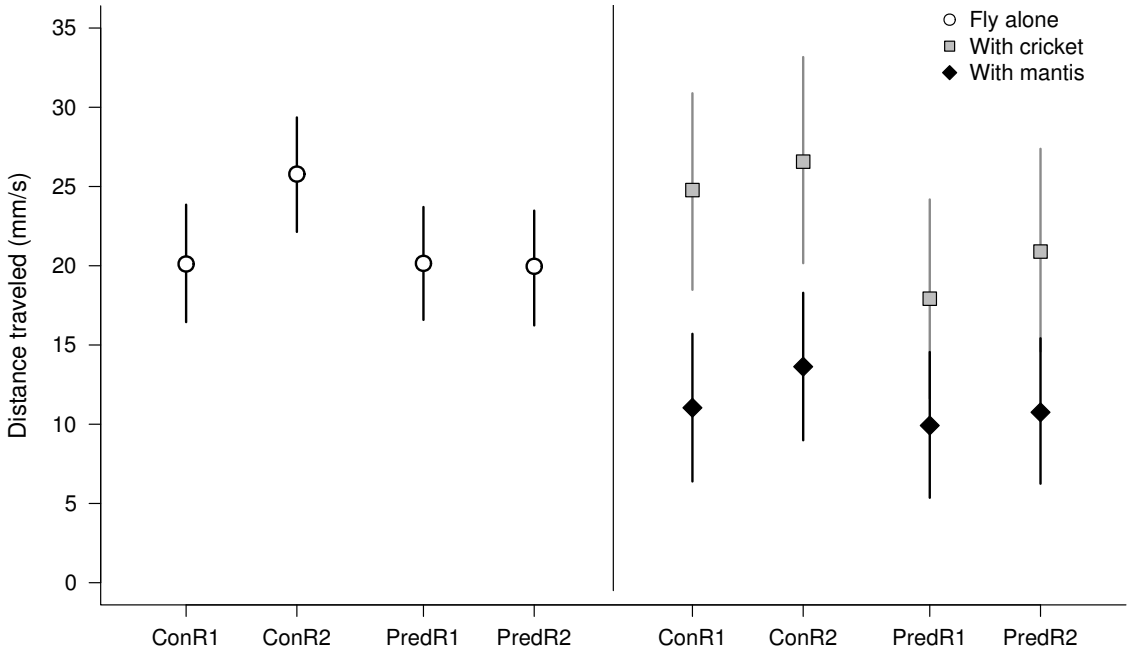

Figure C.4 **Effect of the presence of a predator on the locomotory behavior of *Drosophila melanogaster*** Mean distance traveled by flies per second when alone (white points), with the cricket (grey squares), and with the mantis (black diamonds). Error bars are 95% confidence intervals.

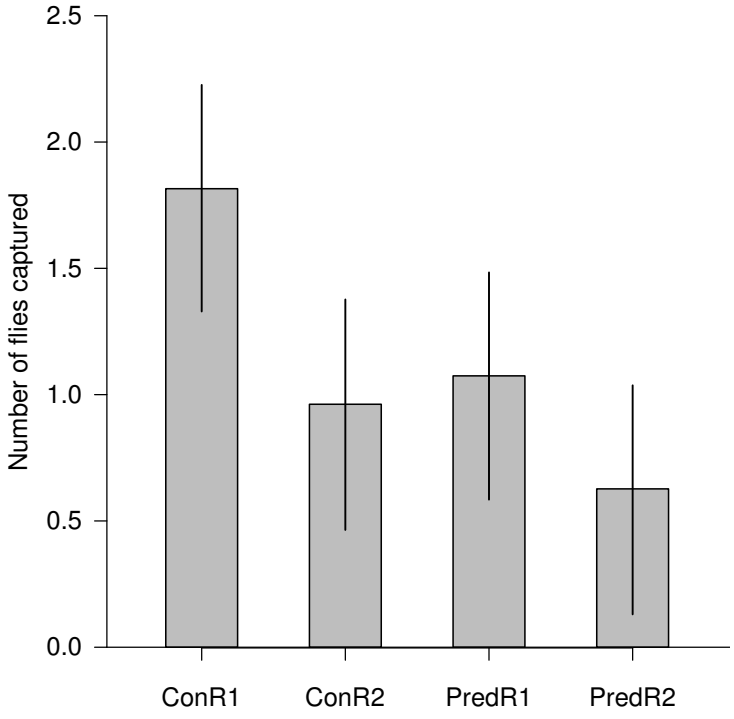

Figure C.5 **Survival ability of control and predation populations** Bars show the mean number of flies captured per 10 minute period. Error bars are 95% confidence intervals.

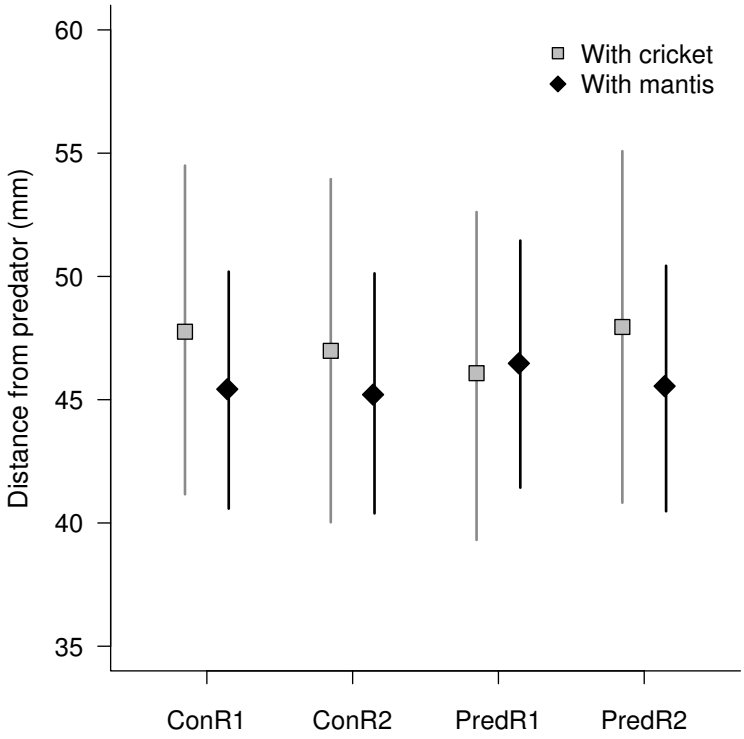

Figure C.6 **Distance between the fly and the predator or control insect** Mean distance maintained between the fly and the cricket (grey squares) or the mantis (black diamonds). Error bars are 95% confidence intervals.

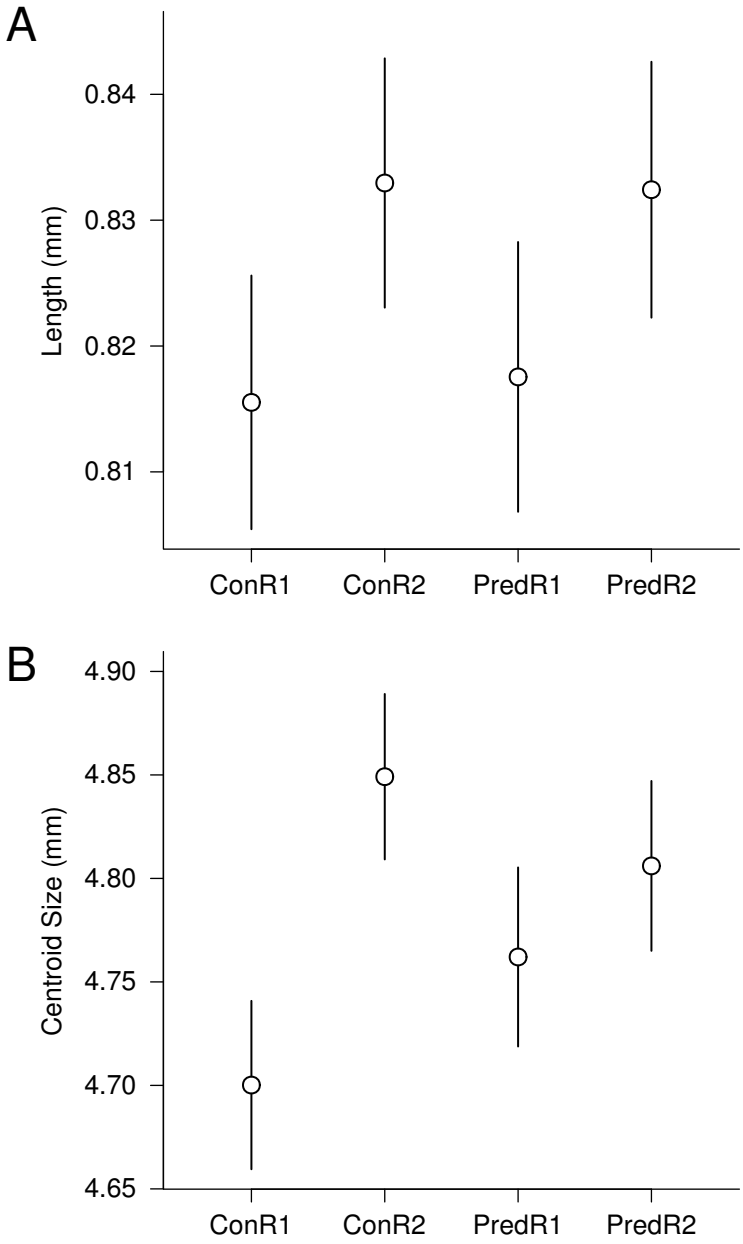

Figure C.7 **Body size and wing size in the evolved populations.** (**A**) Mean thorax length and (**B**) centroid size after 99 generations of evolution. Error bars are 95% confidence intervals.

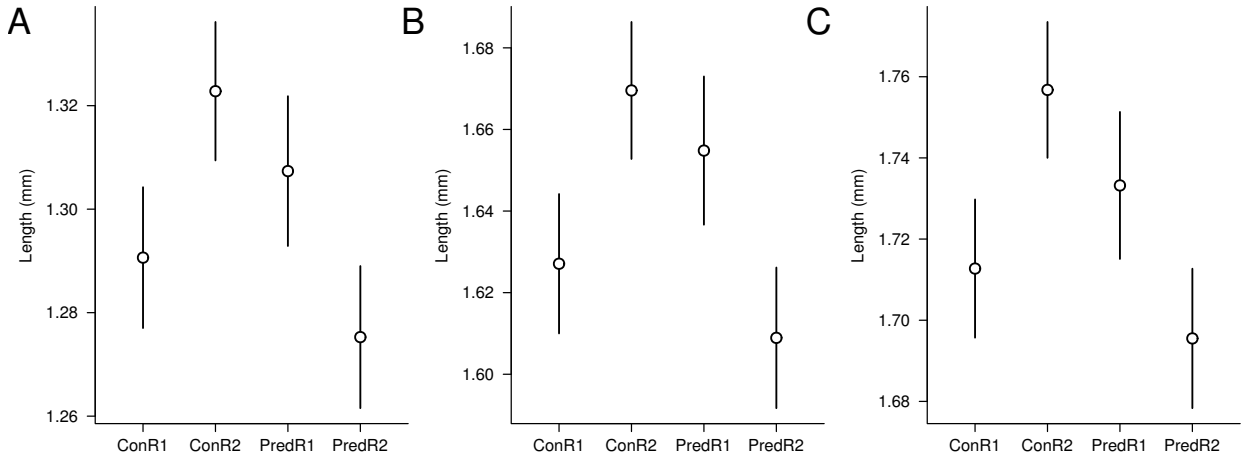

Figure C.8 **Leg lengths in the evolved populations** Mean length for (**A**) leg 1, (**B**) leg 2, and (**C**) leg 3. Error bars are 95% confidence intervals.

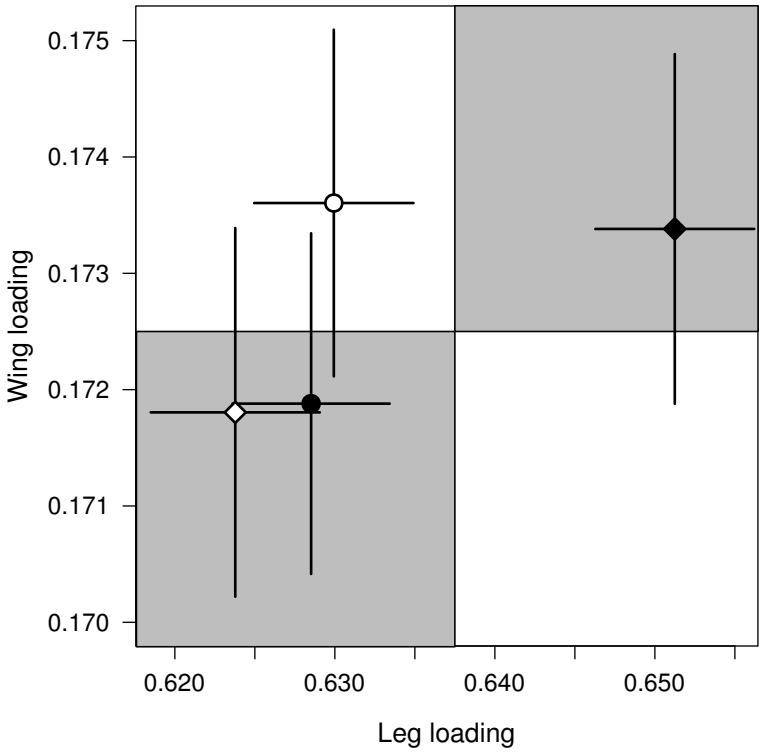

Figure C.9 **Scaling relationships are associated with successful anti-predator strategies.** Correlation between wing loading and leg loading for control (circles) and predation (diamonds) populations. Open symbols denote replicate 1 and filled symbols denote replicate 2. Grey boxes denote quadrants in which flies show increased survival as shown in Figure 4.3. Populations in the lower left quadrant utilize a primarily flying escape strategy. Populations in the upper right utilize a increased dodging strategy. Error bars are 95% confidence intervals.

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