UNIVERSITY OF CALIFORNIA,
IRVINE

Evolutionary Physiology of *Drosophila melanogaster*

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

James Nicholas Kezos

Dissertation Committee:
Professor Michael R. Rose, Chair
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2017

DEDICATION

To

My parents, Jim and Bessie, for always believing in me.

My sister, Nicole, for going on this journey together.

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It has been a long road for me to get here, and without my colleagues, advisors, teachers, friends, and family, it would have definitely been more of a challenge. The past ten years at UC Irvine have been some of the toughest years, but also some of the best years. My second home has granted me this wonderful opportunity to pursue a career as a scientist, filled with many new friendships.

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My formal education may have ended, but the education process never truly ends. With every end to a project or experiment, there is always something new to ask and think about. I cannot wait to see where this road now takes me.

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ABSTRACT OF THE DISSERTATION

Evolutionary Physiology of *Drosophila melanogaster*

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Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2017

Professor Michael R. Rose, Chair

Over the past 30 years of experimental evolutionary research on *Drosophila*, strong functional associations have been established between organismal characters, life history, and behavior. Evolutionary physiologists use stress as a tool, either to measure an organism's physical robustness, or to create differentiated populations with which to study adaptation. However, many questions are unanswered. For example, do short periods of strong selection generate similar levels of functional divergence to those generated by long-sustained selection? And if so, can we take the already advantageous *Drosophila* model system and use it to effectively study vertebrate diseases (i.e. cardiovascular disease and obesity-related disorders).

Chapter 1 examines the relationship between evolutionary history and physiological differentiation. We observed classic physiological characters, specifically stress resistance and locomotion, as well as a character of recent interest, heart robustness. We found that short periods of strong selection applied to outbred Mendelian populations can readily generate high levels of functional differentiation.

Chapter 2 revolves around the interrelationships among major physiological systems. By combining electrical pacing and flight exhaustion assays with manipulative conditioning, we

started to unpack the interrelationships between cardiac function, flight endurance, and stress resistance. One major insight is the adverse impact of lipids on *Drosophila* heart robustness, a parallel result to many comparable studies in human cardiology.

With human obesity growing to epidemic proportions in the United States, and excessive lipid accumulation being a risk factor for heart disease, we sought to observe the effects of lipid accumulation in *Drosophila*. Chapter 3 discusses the effects of intense selection for increased starvation resistance on ten outbred *Drosophila* populations. These populations displayed cardiac dysfunction, increased adult mortality, and elevated lipid levels, making them a useful model system for heart disease and obesity-related disorders. In Chapter 4, I emulated the effects of chronic consumption of the high-fat, high-caffeine fast-food diet by exposing flies to coconut oil and caffeine. Similarly, the findings here continue to support the general inference that high lipid levels present challenges for the *Drosophila* heart. Fruit flies could be an invaluable resource in understanding the molecular, genetic and other machinery underlying heart disease.

INTRODUCTION

Drosophila melanogaster: A Model for Evolutionary Physiology

For the past 40 years, experimental evolution has been a powerful approach to the study of adaptation. A key application of experimental evolution is using a well-defined selection protocol to create a replicated set of populations that have been differentiated relative to its replicated control populations. The value of these populations increases with the ability to compare control and selected population allele frequencies throughout the genome for associations between types of phenotypic differentiation. In short, the tools of experimental evolution allow researchers to conduct strong-inference tests of hypotheses concerning both phenotypic and genetic responses to selection.

A common system for experimental evolution is the complex metazoan model genus, *Drosophila*. The features that make this organism so ubiquitous and attractive for any biologist to work with are its short generation-time, ease of maintenance, and abundant public genomic data (Burke and Rose 2009). Maintaining hundreds of fly populations, each with an effective population size on the order of 10^3, is relatively easy. These populations are large enough to retain abundant genetic variation during selection experiments of moderate duration (vid. Mueller et al. 2013). Because of the ability to maintain *Drosophila* populations with abundant genetic variation, laboratory selection can produce physiological changes quite rapidly, in as few as 10 generations (reviewed in Gibbs 1999). Additionally, the well-developed physiological background information make the fruit fly model especially suited for evolutionary physiology. Thus, the last 30 years have seen appreciable progress in the study of the evolutionary physiology of *Drosophila* species, especially *D. melanogaster* (e.g. Gibbs and Gefen 2009; Huey et al. 1991; Rion and Kawecki 2007; Weber 1996).

Selection on different life-histories has been a keystone theme in *Drosophila* research (Djawdan et al. 1998; Graves and Rose 1990; Graves, Luckinbill and Nichols 1988; Graves et al. 1992; Luckinbill and Clare 1985; Luckinbill et al. 1984, Luckinbill et al. 1988; Rose 1984; Rose et al. 1984; Service et al. 1985; Service 1987; additional articles compiled or reviewed in Rose, Passananti and Matos 2004). In experimentally evolved populations with postponed senescence, a variety of stress resistance and performance characters are improved (Graves and Rose 1990; Graves et al. 1988; Service et al. 1985). Manipulating the age of reproduction not only affects the lifespan of *Drosophila* populations, it indirectly impacts many physiological characters.

I. *Drosophila* Model for Stress Resistance and Metabolic Reserves

The Rose Laboratory's longer-lived $O_{1\text{-}5}$ populations have generally been found to tolerate starvation and desiccation significantly better than their control B populations, which have shorter average lifespans (Service et al. 1985). Results from experiments cited in the previous paragraph suggest the mechanisms underlying stress resistance might also be mechanisms necessary for survival at late ages. In one reverse-selection experiment, the five O populations were returned to their ancestral reproductive schedule. Service et al. (1988) found a decrease in starvation resistance, but not desiccation resistance, in as little as 22 generations of reverse selection. It took a little more than 100 generations of reverse-selection for these reverse-selected lines to see desiccation resistance fall back to the levels of the short-lived lines (Graves et al. 1992). Additional reverse-selection experiments in the Rose Laboratory found similar results. Longevity and starvation resistance dramatically declined as a response to such reverse selection, but desiccation resistance did not respond significantly over about 60 generations (Passananti et al. 2004b). The delayed response of desiccation resistance to reverse

selection for shorter lifespan in $O_{1\text{-}5}$ populations suggests that increased desiccation resistance evolves in populations selected for postponed aging, but at relatively little cost for early reproduction. Because starvation resistance falls rapidly in reverse-selected lines in these same experiments, this trait appears to trade-off powerfully with benefits associated with early reproduction (Service and Rose 1985).

Selection for increased starvation and desiccation resistance produces rapid responses within populations of *D. melanogaster* (Archer et al. 2007; Chippindale et al. 1996; Harshman and Schmid 1998; Rose et al. 1992). Direct selection for increased starvation (Rose system "SO" and "SB" populations) and desiccation (Rose system "D" populations) resistance results in a correlated increase in longevity and decreased fecundity (Rose et al. 1992). Decreased pre-adult viability and slower development time was seen in populations directly selected for desiccation resistance relative to control populations (Chippindale et al. 1996; Chippindale et al. 1998; Harshman et al. 1999). Overall, this body of work appears to support the hypothesis of trade-offs connecting resource acquisition during larval stages, adult stress resistance, and life history.

Additional reverse selection experiments focused on stress resistance have reinforced some, but not all, of these correlative observations. A variety of differentiated populations created by laboratory natural selection were reverse-selected to their ancestral condition, including populations that had been specifically selected for increased starvation resistance (Teotonio and Rose 2000). Development time, age-specific fecundity, and dry body weight measurements returned to the average values of ancestral populations after 50 generations of reverse-selection. Relaxing selection in populations previously selected for starvation and desiccation resistance led to a significant fall of starvation resistance, but not desiccation

resistance (Passananti et al. 2004a). Nonetheless, these direct selection and reverse-selection experiments suggest a positive correlation between starvation and desiccation resistance, on one hand, and longevity, on the other.

Increasing lipid content in adult flies has been well documented as a candidate mechanism underlying increases in starvation resistance. Populations selected for increased starvation resistance show that lipid content accounts for almost all variation in starvation resistance (Chippindale et al. 1996; Harshman et al. 1999). Pseudocomparative analysis of starvation resistance and lipid content in Rose Laboratory populations provides corroborative results characterizing the starvation-resistance response. The longer-lived $O_{1\text{-}5}$ populations have larger lipid stores than shorter-lived $B_{1\text{-}5}$ populations (Chippindale et al. 1998; Service 1987). Djawdan et al. (1998) found that, in a wide range of experimentally evolved fly populations, the total amount of stored calories in the fly body served as an excellent predictor of starvation resistance.

While starvation resistance appears to be tied to energy in the form of stored lipid and carbohydrates, the physiological basis of desiccation resistance is more complex. Nghiem et al. (2000) found that neither lipid content or total energy content was significantly correlated with desiccation resistance across selected populations. They did find that flies from the short-lived $B_{1\text{-}5}$ populations lost water significantly faster than flies from the long-lived $O_{1\text{-}5}$ lines. However, the water contents of these two sets of populations were not substantially different. Deckert-Cruz et al. (1997) found that carbohydrate content was significantly correlated with desiccation resistance in female flies, but not male flies, from desiccation resistant populations. Both direct selection (Gibbs et al. 1997) and pseudocomparative work (Deckert-Cruz et al. 1997) suggest that flies selected for desiccation resistance accumulate glycogen to sequester water. Further

analysis of desiccation resistant populations indicated that the best predictors for desiccation resistance are characters regulating water balance: rates of water loss and overall water content (Folk et al. 2001; Gibbs et al. 1997; Williams and Bradley 1998). These two factors underpinning desiccation resistance were shown to evolve separately from each other when sustained strong selection for desiccation resistance is applied (Archer et al. 2007).

Enhancing storage of lipid, carbohydrate, and water can increase the stress resistance of flies and promote longevity. The outcomes of direct selection for these stress resistance characters implicate the same lower-level mechanisms that underlie starvation and desiccation resistance in lines selected for life history traits, such as later fecundity. However, at very high levels of stress resistance, this pattern of correlation between stress resistance and life history breaks down. Phelan et al. (2003) provides evidence for the disappearance and even reversal of the commonly seen positive correlations between selection for stress resistance and longevity, in the same system of populations. They suggest that the long period of sustained selection led to this breakdown. More direct laboratory selection for extreme values of stress resistance can also lead to a breakdown in the correlation between stress resistance and longevity (Archer et al. 2003). Although long-term selection produces substantial enhancements in functional characters, correlations between certain characters may breakdown as a result of still other factors, such as the base population used to initiate selection (Rose et al. 2005).

II. *Drosophila* Model for Heart Robustness and Disease

The tube-like heart structure in *Drosophila* separates this model species from other common invertebrate model species, making *Drosophila* the simplest model organism that can be used for heart function studies (Bier and Bodmer 2004). Over the past 30 years, *Drosophila*

has become the invertebrate system of choice to study heart development, function, and aging, as well as obesity-related disorders. The conserved mechanisms of heart development and function between *Drosophila melanogaster* and vertebrates provides further support for the appropriate use of this model for heart research (Bodmer 1995; Bodmer and Frasch 1999; Bodmer et al. 2005; Cripps and Olsen 2002; Harvey 1996;). Additionally, the dorsal vessel of *D. melanogaster* is homologous to the vertebrate heart and pumps hemolymph rhythmically (Bodmer and Venkatesh 1998; Curtis et al. 1999; Rizki 1978). Lastly, there is extensive information available concerning *D. melanogaster* heart anatomy (Curtis et al. 1999; Dulcis and Levine 2005; Miller 1950; Rizki 1978) as well as gene activation and molecular signaling during morphogenesis in heart embryology (Cripps et al. 1999; Gajewski et al. 2000; Molina and Cripps 2001).

There are many parallels in heart development, function, and disease between Drosophila and vertebrates. Bier and Bodmer (2004) and Piazza et al. (2009) showed that *Drosophila* cardiac dysfunction from electrical pacing increases with adult age, suggesting a decline in heart robustness during aging. Additional studies at the Burnham Institute have shown cardiac arrhythmia increases with adult age in *Drosophila* (Ocorr et al. 2007; Wessells and Bodmer 2004). But the research connections between fruit fly hearts and human hearts are deeper than functional parallels. Despite the anatomical and morphological differences in the heart structure of *Drosophila* and vertebrates, there are numerous parallels in the embryological and molecular mechanisms underlying early heart development (Bodmer 1995; Bodmer and Venkatesh 1998; Bodmer et al. 2005; Wolf and Rockman 2011).

The *D. melanogaster* heart has been used as a model for identifying genes that cause heart disease (e.g. Wolf et al. 2006). The genetics of heart formation and disease in vertebrates are conserved in *Drosophila* genetics. For example, *Drosophila* heart formation is dependent on

the gene *tinman* (Bodmer 1993). Without the expression of *tinman*, not only does the heart not form, but other visceral and dorsal skeletal muscles do not form (Bodmer and Venkatesh 1998). In several vertebrate species, two genes related to *tinman* are *Nkx2-3* and *Nkx2-5*. However, the dysfunction created via knockout mutations of these vertebrate genes occurs later in the developmental stages of the heart. The vertebrate heart tube fails to loop properly due to the lack of (1) bHLH protein eHAND (Biben and Harvey 1997) and (2) the Ankyrin-repeat protein CARP (Zou et al. 1997). Vertebrate *Nkx2-5* mutants do not suffer a complete absence of heart formation, unlike *Drosophila tinman* mutants. Other vertebrate genes and transcription factors associated with heart function and development that are conserved are: *MEF2 genes*, *BMP* genes, bHLH proteins, and GATA transcription factors (reviewed in Bodmer and Venkatesh 1998).

While some cases of human heart disease arise from such single-locus mutations, such as cases that arise from *opa1* (Shahrestani et al. 2009), other types of heart disease may involve many genes and thus many biochemical pathways. Heart disease that is common among present-day human populations is unlikely to result from deleterious alleles of major effect. It would be unlikely for these alleles to rise to high frequencies in outbred populations, because of natural selection acting to remove them. It is therefore useful to study heart function in large outbred populations of *D. melanogaster* that differ in allele frequencies at many loci, rather than just at single loci of large effect. Such populations can be produced through experimental evolution in which specific selection protocols are used to produce phenotypic divergence (Garland and Rose 2009; Teotonio et al. 2009).

Human obesity has grown to epidemic proportions in the United States, with excessive lipid accumulation being a risk factor for metabolic disorders and heart disease. Two approaches

can be taken to study cardiovascular disease and other obesity-related disorders in *Drosophila*: (1) exposing populations to high-fat or high-sugar diets (e.g. Birse et al. 2010), or (2) creating a set of obese populations via selection (e.g. Hardy et al. 2015). In Birse et al. (2010), fruit flies fed a high-fat diet had (i) increased overall triglyceride fat content, (ii) increased cardiac lipid accumulation, (iii) reduced cardiac contractility, (iv) blocked heart electrical conduction, and (v) severe structural pathologies. They found that either overexpressing lipase or reducing insulin-TOR activity can lower lipid accumulation during the high-fat diet exposure. Reduced lipid accumulation in turn lessens the adverse effects of high-fat diet on cardiac fat accumulation and cardiac function.

We envision a future for evolutionary physiology that is infused with experimental evolution and multi-omic tools, to connect physiological performance, life history, and gene expression on the phenotypic level with polymorphism at the genotypic level. This dissertation extends the study of the evolutionary physiology of *Drosophila*, with a focus on heart disease. It is also intended to provide useful phenotypes for the application of recent genome-wide findings concerning the *Drosophila* populations of the Rose laboratory (e.g. Graves et al. 2017) to the mechanistic analysis of *Drosophila* physiology.

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CHAPTER 1

Convergent functional evolution in *Drosophila melanogaster*

I. Abstract

An important issue in evolutionary physiology is the evolutionary history of the organisms being studied. This study compares 30 populations of *Drosophila melanogaster* that descend from a common ancestral laboratory population. These populations have extensively and reproducibly diverged with respect to multiple life-history and physiological characters. Fifteen of these populations have been continually subjected to one of three distinct selection regimes for at least 20 years, in three groups of five replicate populations. The other 15 populations have been recently subjected to these same three distinct selection regimes, again as replicate groups of five populations, for six years or less. All 30 populations were compared for four physiological characters: desiccation resistance, starvation resistance, flight endurance, and resistance to electrical cardiac pacing. Some comparisons of these characters revealed partial failures of evolutionary convergence to the same average level of functional performance, when the recently-imposed and the long-imposed terminal selection regimes were the same. But in most cases, recently imposed selection regimes mattered more than deeper evolutionary history for the evolutionary physiology of *Drosophila* populations. Furthermore, these findings suggest that short periods of strong selection applied to outbred Mendelian populations can readily generate functional differentiation for the purpose of physiological analysis. Long-sustained selection appears to be unnecessary.

I.Introduction

Experimental evolution is a powerful technique for generating extensive functional differentiation (Garland and Carter 1994). While experimental evolution requires an initial investment of sustained and replicated selection (vid. Garland and Rose 2009), functional studies of experimentally evolved populations benefit from their extensive differentiation combined with known phylogenetic histories that are founded on well-defined selection regimes (Burke and Rose 2009; Swallow et al. 2009).

A common system for experimental evolution is the complex metazoan model genus, *Drosophila.* The short generation-time and ease of maintenance of *Drosophila* populations, the available genomic data, as well as well-developed background information, make the fruit fly model especially suited for the study of functional biology. Thus the last 30 years have seen appreciable progress in the functional parsing of *Drosophila* species, especially *D. melanogaster* (e.g. Huey, Patridge and Fowler 1991; Gibbs and Gefen 2009; Rion and Kawecki 2007; Weber 1996).

A common theme of *Drosophila* experimental evolution has been selection for very different life-histories (Djawdan et al. 1998; Graves and Rose 1990; Graves, Luckinbill and Nichols 1988; Graves et al. 1992; Luckinbill and Clare 1985; Luckinbill et al. 1984, Luckinbill et al. 1988; Rose 1984; Rose et al. 1984; Service et al. 1985; Service 1987; additional articles compiled or reviewed in Rose, Passananti and Matos 2004. Early on it was found that a variety of stress resistance and performance characters (e.g. starvation resistance and flight duration) were improved in experimentally evolved stocks with postponed senescence (Graves and Rose 1990; Graves et al. 1988; Service et al. 1985). Additional selection experiments then showed that some stress resistance characters, such as resistance to ambient ethanol or desiccation,

evolve in parallel with each other, while starvation resistance evolves independently of those two types of stress resistance (Graves et al. 1992; Service, Hutchinson and Rose 1988).

Further work has unpacked some of the mechanistic specifics underlying these evolving functional characters. Adult starvation resistance, for example, depends overwhelmingly on total stored calories (e.g. Djawdan et al. 1998), while desiccation resistance depends chiefly on water content and rate of water loss (e.g. Gibbs, Chippindale and Rose 1997). These two factors underpinning desiccation resistance were further shown to evolve separately from each other when sustained strong selection for desiccation resistance is applied (Archer et al. 2007). Overall, we would say that the combination of functional analysis and experimental evolution has allowed the study of the functional basis of fruit fly life history to make rapid progress.

The question we focus on here is the relationship between evolutionary history and functional differentiation. We examined both classic functional characters that have long been studied in *Drosophila*, specifically stress resistance and locomotion, as well as a functional character of recent interest, heart robustness. The *Drosophila* heart is a vital physiological system that interacts with flight musculature, metabolism, and respiration to allow survival and locomotion in stressful environments. The system we employed features 30 *D. melanogaster* populations: ten populations selected for accelerated development (A-type); ten populations that have been cultured using the same protocols as the ancestral population which serves as the "base" for our entire experimental evolution system (B-type); and ten populations selected for delayed reproduction (C-type). Of these 30 populations, 15 populations (five populations for each selection regime) were subjected to A, B, or C-type selection for 300-1000 generations immediately prior to the physiological experiments reported here, all for more than 20 years. The other 15 populations were subjected to these selection regimes for just 30 to 200 generations

prior to our experiments, over periods of no more than 6 years. All these populations share the same ancestral "IV" population that was established in the laboratory in 1975. In effect, these two sets of 15 populations differ strikingly with respect to their *intermediate* evolutionary histories, as both their common ancestor and their most recent selection regimes are parallel among the three A, B, and C selection regimes. We will use them to show that, relatively speaking, recently imposed selection regimes matter more than somewhat deeper evolutionary history for functional differentiation, at least within the *Drosophila* experimental evolution paradigm.

III. Materials and Methods

3.1 Populations Used

This study employed 30 of the large, outbred, and highly differentiated populations created by the Rose laboratory over more than 30 years (Rose 1984; Rose et al. 1992; Rose et al. 2004). These populations have been selected for reproduction at different ages in the life cycle: 10 days (5 ACO populations, 5 AO populations), 14 days (5 B's, 5 BO's), and 28 days (5 CO's, 5 NCO's). [See Chippindale et al. (1997) for details concerning the creation of ACO stocks; Rose (1984) for the B stocks; Rose et al. (1992) for the creation of the CO stocks; Burke (2010) for the AO, BO, and NCO stocks.] All of the populations assayed here descend from a single *Drosophila melanogaster* population, called IV. The B_{1-5} populations (baseline) and O_{1-5} (10-week generation cycle) were derived from the single IV population in 1980. The ACO_{1-5} populations, which "A" stands for accelerated, were derived from the CO_{1-5} populations. The CO_{1-5} populations were derived from the O_{1-5} populations. The AO_{1-5}, BO_{1-5}, and NCO_{1-5} populations were all derived from the O_{1-5} populations. Each new population was founded from

the same number replicate of their ancestral population. All 30 populations were kept at large effective populations sizes (n > 1,000) to avoid confounding inbreeding effects. The ACO, B, and CO populations are referred to as "long-selected" lines because they have been undergoing their current selection regimes since 1991, 1980, and 1989, respectively. The AO, BO, and NCO populations are referred to as "recently derived" because these populations have been undergoing their current selection regimes since 2008, 2007, and 2009, respectively. The specific evolutionary history for this experimental system is shown in Fig. 1.1.

It is important to note that, though populations with different life-cycle timing are not specifically selected for increased stress resistance, they do nonetheless show differences for a variety of stress and performance characters (vid. Graves and Rose 1990; Graves et al. 1992; Rose et al. 2004; Service et al. 1985).

3.2 Assay Methods

In this study we examined four key functional characters: desiccation resistance, starvation resistance, flight duration, and heart function. The following are the specific assay procedures used.

Rearing Protocols

Two run-in generations, the first being 12 days and the second being 14 days, were used to remove any parental or grand-parental epigenetic effects. The populations were cultured in regular banana-molasses food from egg to adult, on a 24L:0D light schedule. Populations were maintained and assayed in a laboratory kept at 23 – 25 °Celsius. Eggs were collected at a density of 60 to 80 eggs per vial after adults were allowed 24 hours to lay eggs. At the end of

each run-in generation (day 12 and day 14), the populations were transferred to an acrylic cage. Replicate populations of the same number (e.g. ACO_1, B_1, CO_1) were handled in parallel at all stages. On day 14 of the second run-in generation, the adults were assigned at random to one of the four physiological assays.

Desiccation Resistance Assay

On day 15 from egg, 30 female flies from each replicate per stock were placed in their own desiccant straws, one fly per straw. A piece of cheesecloth separated the fly from the pipet tip at the end of the straw that contained 0.75 grams of desiccant. The pipet tip containing desiccant was sealed with a layer of Parafilm. Mortality was checked hourly, using lack of movement under provocation as a sign of death.

Starvation Resistance Assay

On day 14 from egg, 20 couples (one male and one female) were placed in individual vials as pairs from each replicate per stock. These vials were filled with about a half an inch of an agar and water medium. Mortality was checked every 4 hours, using lack of movement under provocation as a sign of death.

Flight Exhaustion Assay

On day 15 from egg, 30 to 40 female flies from each replicate per stock were selected at random. The flies were briefly anesthetized using cold-shock by partially submerging a plastic vial with female flies into ice for one to two minutes, and then tethered singly to a monofilament string using Duco cement glue applied to the mesonotum region of the thorax. The flight

response was stimulated by tapping the tethering string, with total flight duration being recorded. Flight was terminated if the fly could no longer be made to resume flight by tapping within a continuous 3-minute interval, or at least seven brief flights were attempted but not sustained consecutively during that period. The protocol for this assay is described further in Graves et al. (1988).

Cardiac Pacing Assay

On day 14 from egg, 40 female flies from each replicate population were chosen at random to undergo cardiac pacing. The cardiac pacing assay was similar to the approach described by Wessells and Bodmer (2004). The flies were anesthetized using tri-ethyl amide, also known as FlyNap, and then placed on a microscope slide prepared with foil and two electrodes. Two electrodes were attached to a square-wave stimulator in order to produce electric pacing of heart contraction. Anesthetized flies were attached to the slide between the foil gaps using a conductive electrode jelly touching the two ends of the fly body, specifically the head and the posterior abdomen tip. The shocking settings for this assay were 40 volts, 6 Hertz, and 10 ms pulse duration. Each shock lasted for 30 seconds. An initial check of the status of the heart was made after completion of the shock, followed by a check after a 2-minute "recovery" period. Heart status was scored as either contracting or in cardiac arrest. The rate of cardiac arrest was used as an indicator of heart robustness.

2.3 Statistical Methods

Each of the three measured phenotypes, starvation resistance, desiccation resistance, and flight exhaustion were analyzed separately. The only difference between these analyses was the

inclusion of both sexes in the starvation measurements while only females were used in the desiccation and flight assays. We outline the linear mixed effects model used for starvation resistance. Removal of the effects of sex from this model yields the models used for desiccation and flight.

Let y_{ijklm} be the measured starvation resistance for selection regime-*i* (*i*=1 (A type), 2 (B type), and 3 (C type)), selection duration-*j* (*j*= 1 (recent), 2 (old)), sex-*k* (*k*=1 (female), 2 (male)), population-*l* (*l*= 1..30), and individual-*m* (*m*=1..n_l). Then the effects of the fixed and random effects can be modeled as,

$$y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_i\delta_j\pi_{ij} + \delta_i\delta_k\theta_{ik} + \delta_j\delta_k\varphi_{jk} + \delta_i\delta_j\delta_k\omega_{ijk} + b_l + \varepsilon_{ijklm}$$

where δ_s=0 if *i*=1, and 1 otherwise. The main effects of selection regime, selection duration and sex are measured by α, β, and γ respectively. Interactions between pairs of these three parameters are measured by π, θ, ϕ, while the three way interactions are measured by ω. The different populations contribute random effects to these measurements by genetically based differences that arise due to random genetic drift and are measured by *b* while individual random variation is measured by ε. Both sources of random variation are assumed to be independent normally distributed random variables with zero means. The model parameters were estimated with the R *lme* function (R Core Team, 2015). Various pairwise tests of the model predictions were used to test for fixed effects differences using the R *lsmeans* function. Type-I error rates for multiple testing by *lsmeans* was controlled by Tukey's studentized range method (Miller 1966).

Cochran-Mantel-Haenszel (CMH) tests were used to analyze the rates of cardiac arrests between two different stocks (i.e. ACO_i vs AO_i). The CMH test is used when there are repeated

tests of independence, or multiple 2x2 tables of independence. This is the equation for the CMH test statistic, with the continuity correction included, that we used for our statistical analyses:

$$X^2_{\mathrm{MH}} = \frac{\{|\Sigma\left[a_i - \frac{(a_i + b_i)(a_i + c_i)}{n_i}\right]| - 0.5\}^2}{\Sigma(a_i + b_i)(a_i + c_i)(b_i + d_i)(c_i + d_i)/(n_i^3 - n_i^2)}$$

We designated "a" and "b" as the number of cardiac arrests in population *i* of the first stock and population *i* of the second stock, respectively. We designated "c" and "d" as the number of contracting hearts in the two populations. The n_i represents the sum of a_i, b_i, c_i, and d_i. The subscript *i* (i = 1..5), representing one of the five replicate populations within each of the six stocks.

IV. Results

4.1 Starvation Resistance

As in previous studies (e.g. Djawdan et al. 1998), populations with a longer generation cycle were observed to have relatively increased starvation resistance in both females and males (Figs 1.2 and 1.3, respectively). The A-type populations had the shortest starvation survival times, while the B-type populations had intermediate starvation survival times, and the C-type populations had the longest starvation survival times, for both males and females. In females, the three selection regimes all significantly differentiated from each other on a pairwise basis. In males, the A-type and B-type populations did not significantly differ in starvation resistance (see Fig. 1.3). The males in the C-type populations did have significantly higher survival times than the A-type and B-type populations on a pairwise basis. When analyzing the three pairwise differences between long-established and recently derived populations within selection regimes

in both males and females, there are no statistically significant differences for starvation resistance (e.g. the average ACO female starvation time is 31.4 hours while the AO average is 30.9 hours, $p > 0.9035$).

4.2 Desiccation Resistance

The mean desiccation resistance of the 30 populations for females (Fig. 1.4) indicates a similarity in desiccation resistance between A-type and B-type populations, with the C-type populations being superiorly resistant. The C-type populations, with their longer life-cycles, were observed to have increased desiccation resistance, as in previous studies (e.g. Graves et al. 1992; Rose et al. 1992). The A-type and B-type populations did not significantly differ in desiccation resistance, and they collectively had the shortest desiccation survival times. The C-type populations did have significantly higher survival times than the A-type and B-type populations on a pairwise basis (see Table 1.1). In addition, when analyzing the three pairwise differences between long-established and recently derived populations within selection regimes, there are no statistically significant differences for desiccation resistance (e.g. the average ACO female desiccation time is 9.07 hours while the AO average is 9.13 hours, $p = 0.9208$).

4.3 Flight Duration

Populations with a longer generation cycle were observed to have longer flight duration times, as in previous studies (e.g. Graves et al. 1988; Graves et al. 1992). The A-type populations had the shortest average flight duration time, while the B-type populations had intermediate average flight duration time and C-type populations had the longest average flight duration time (see Fig. 1.5). The three selection regimes all significantly differentiated from

each other on a pairwise basis (see Table 1.1). When analyzing the three pairwise differences between long-established and recently derived populations within selection regimes, there are no statistically significant differences for flight duration in two of the three comparisons. There was a slight significant difference (p-value: 0.0409) between the average ACO female flight duration time (21.97 minutes) and the average AO female flight duration time (30.9 minutes).

4.4 Cardiac Arrest Rate

Populations with longer generation cycles were found to have lower cardiac arrest rates. The A-type populations had the highest cardiac arrest rate, the B-type populations had an intermediate cardiac arrest rate, and C-type populations had the lowest cardiac arrest rate (Fig. 1.6). The three selection regimes all significantly differentiated from each other on a pairwise basis, as determined by using the Cochran-Mantel-Haenszel test. When analyzing the three pairwise differences between long-established and recently derived populations within selection regimes, there are no statistically significant differences for cardiac arrest rates (e.g. the average ACO cardiac arrest rate is 56.5% while the AO average is 54%, p-value = 0.688).

V. Discussion

5.1 Functional Differentiation Among Selection Regimes

Based on the statistical analysis of our results, we find clear differentiation for all our characters among the A, B, and C selection regimes, although not all pairwise comparisons between selection regimes were statistically significant. For example, while desiccation resistance for CO and NCO were significantly higher than the A- and B-type populations,

pairwise comparison of desiccation resistance between A and B type populations did not reveal statistically significant differentiation.

For starvation resistance, desiccation resistance, and flight duration, the A-type populations were generally inferior to the B-type and C-type populations. For these three functional characters, the B-type populations were intermediate, with the C-type populations either surviving the longest in a stressful environment, or having the longest flight endurance. There are two exceptions to this conclusion: (1) lack of statistical differentiation in male starvation resistance between A-type and B-type populations, and (2) lack of statistical differentiation in desiccation resistance between A-type and B-type populations. These results parallel those found for life-history differences among these 30 populations (Burke et al. 2016). They are also in keeping with previous studies of stress resistance (e.g. Djawdan et al. 1998), flight duration (Graves et al. 1992), and heart pacing (Shahrestani 2011) for comparable populations with respect to life-history differentiation. Specifically, the longer the generation cycle (e.g. 28 days for C-type), the more "successful" the populations were during our tests. Our lab has published multiple studies with qualitatively similar results to these (collected or reviewed in Rose et al. 2004).

5.2 Long-Standing Versus Recently-Derived Populations

Having replicated the qualitative patterns of past studies, the key question remains: what is more important for the differentiation of these 30 populations, their most recent selection regime or their deeper evolutionary history? Within each of the A, B, and C sets of ten populations, we have five populations that have been long-selected for the latest, or distal,

selection regime and a set of five populations which have been subjected to the same latest, or distal, selection regime for far fewer generations.

Not only did we see a clear trend of differentiation between the three selection regimes in the majority of the functional characters, we also found rapid convergence within the ten populations sharing the same terminal selection regimes, despite radical differences in the number of generations of selection under that particular regime. In effect, it took a hundred generations or less to achieve almost the same level of functional differentiation as hundreds to a thousand generations of the same type of selection. For example, the pronounced differences in the intermediate evolutionary histories of the long-standing and new B-type populations had remarkably little effect on their ultimate convergence, after more than 25 years during which they had markedly different selection regimes. There is one within selection regime pairwise comparison that achieves moderate statistical differentiation; the flight duration between the ACO and AO populations (p-value = 0.0409).

5.3 Mechanistic possibilities

What we have observed in this study is corroboration of previous findings with regard to stress resistance and flight, both those from our lab as well as studies from outside our lab (cf. Gibbs and Gefen 2009; vid. Rose et al. 2004;). Our expectation is that the underlying biochemical mechanisms behind these functional foundations for life-history are likely to be the same mechanisms as those found previously. For example, total stored calories have been strongly correlated with starvation resistance among a greater range of types of populations than we have studied here (Djawdan et al. 1998), whereas trehalose and glycogen content are predictive for desiccation resistance and flight duration (Djawdan et al. 1998; Graves et al.

1992). In addition to carbohydrate content, decreasing water loss rates and increasing bulk water content are two mechanisms which evolve in response to selection for desiccation resistance (Archer et al. 2007; Gibbs et al. 1997). In other words, our view is that selection for the three different life-cycles of our 30 population system has probably produced the same hierarchies of mechanistic differentiation that we have found before. However, we will be testing this prediction in future studies.

The more novel physiological findings reported here concern the fruit fly heart. The differences that we detected in heart robustness among the three selection regimes could depend on multiple underlying mechanisms. The A-type populations have been selected for accelerated development; they age faster and are significantly smaller than both B-type and C-type populations (Burke et al. 2016; Chippindale, Alipaz and Rose 2004). Prior studies have shown that our A-type populations have reduced levels of lipids, proteins, and carbohydrates compared to the B and C-type populations, with the C-type populations having the highest levels of these macromolecules (vid. Rose et al. 2004). The A-type populations have a significantly higher rate of cardiac arrest after pacing compared to both the B-type and C-type populations. The A-type and C-type populations have an 18-day difference in their generation times, whereas the A-type and B-type populations have a 5-day difference. Despite only a 5-day difference in generation time, it is evident that the B-type populations are intermediate with respect to cardiac robustness.

What underlying mechanisms could account for this spectrum of heart robustness? In an experiment looking at the effect of triglycerides, glycogen and water content on heart properties, it was determined that differences in the level of these macromolecules do not significantly affect cardiac robustness in populations with 3 or 4 week life cycles (Shahrestani 2011). This suggests that body content differences are not as important as life-history differentiation in determining

cardiac robustness. On a still larger scale than the study of Shahrestani, we show that longer-lived C- type populations have increased rates of heart recovery after pacing compared to shorter-lived A-type populations. However, additional experiments in our lab (unpublished), and experiments from various colleagues that selected for starvation resistance (Hardy et al. 2015) or exposed *Drosophila* populations to a high fat diet (Birse et al. 2010), have found a positive correlation between increased body triglycerides and cardiac dysfunction.

5.4 Practical Implications for Functional Research

Our laboratory has imposed some of our selection regimes for as many as 1,000 generations, particularly the B selection regime. This has involved sustaining the same rearing protocols, and replicate-population separation, over decades. The present results suggest that it may not be necessary to do so for the study of functional biology. Over less than 200 generations of selection, the five recently derived A-type populations have evolved similar physiological performance as that produced by more than 800 generations of A-type selection in the five long-selected populations, with one exception: flight endurance. Even more striking is the functional convergence of the recently derived C-type populations, after less than 56 generations of C-type selection, with the long-established C-type populations which had undergone more than 300 generations of C-type selection at the time of our experiments.

For functional convergence to occur in such short periods of evolutionary time, selection requires abundant genetic variation to work with. Abundant genetic variation is possible when maintaining populations with the moderately large estimated effective population sizes characteristically achieved in our system (Mueller et al. 2013), as genome-wide results revealing abundant heterozygosity in A-type and C-type populations suggest (Burke et al. 2010).

Apparently with outbred populations of Mendelian model organisms like ours, it is simply unnecessary to sustain selection for decades to produce experimental material that can be usefully employed in functional studies.

VI. Acknowledgments

We thank James W. Hicks and Timothy J. Bradley for helpful discussions and comments on the experiment. We thank David D. Momtaz for the design and introduction of the electrical pacing assay to our lab, as well as the discussions and insights he provided concerning its use. We are also grateful to the many undergraduate research students who contributed to the stock maintenance and experimental assays, especially Jose Buenrostro, Grigor Azatian, Jerry Harb, Punjot Singh Bhangoo, Annie Khong, Jennifer Majdick, Thao Le, Kathleen Mendoza, and Gabriel Reyes.

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VIII. Tables

Table 1.1. P-values for pairwise stock comparisons, both "between" and "within" selection regime.

Stock Comparison	Male Starvation Resistance	Female Starvation Resistance	Desiccation Resistance	Flight Endurance	Cardiac Arrest Rates
ACO-AO	0.2755	0.9035	0.9208	0.0409	0.688
ACO-B	0.367	0.0099	0.9375	<.0001	0.00004
ACO-CO	<0.0001	<0.0001	<0.0001	<.0001	3.22×10^{-10}
AO-BO	0.6098	0.0001	0.5157	<.0001	0.00503
AO-NCO	<0.0001	<0.0001	<0.0001	<.0001	2.15×10^{-10}
B-BO	0.4935	0.1127	0.3921	0.4909	0.472
B-CO	<0.0001	<0.0001	<0.0001	<.0001	0.036
BO-NCO	<0.0001	0.0002	<0.0001	0.0003	0.0058
CO-NCO	0.8733	0.2704	0.9302	0.0757	0.967

Note: The male starvation resistance, female starvation resistance, desiccation resistance, and flight endurance data were fitted to linear mixed effects models using the R *lme* function. Cochran-Mantel-Haenszel test was applied to the electrical cardiac pacing data.

IX. Figures and Figure Legends

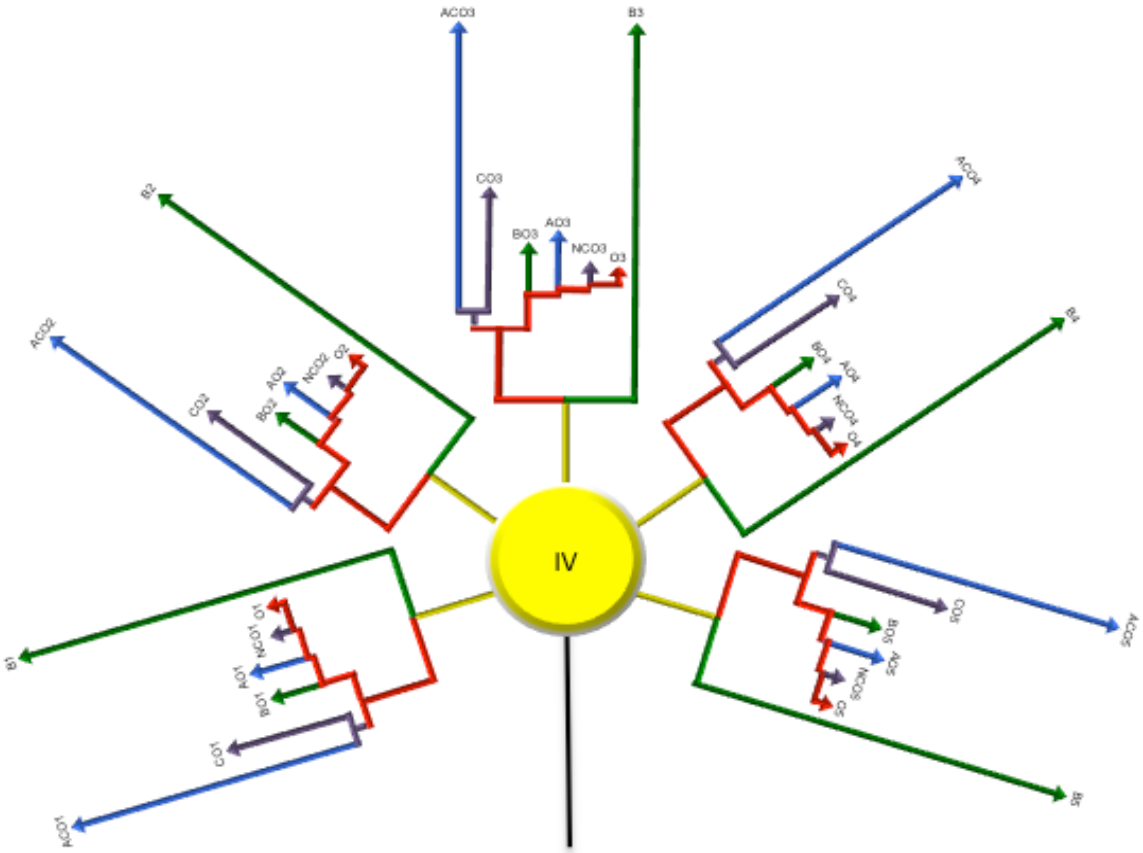

Figure 1.1. Phylogeny of the core *Drosophila melanogaster* model system of the Rose Laboratory. This phylogeny includes six sets of outbred, five-fold replicated populations, as well as the ancestral IV population. Each set of populations undergoes parallel evolution, with little to no migration occurring between replicates.

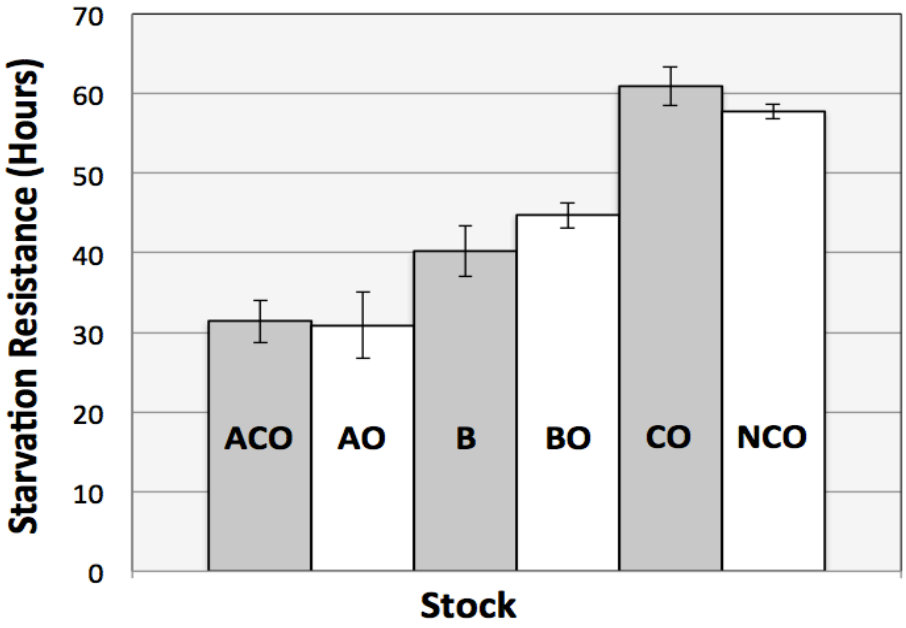

Figure 1.2. Histogram of the average female starvation resistance (hours) for each of the six sets of populations. The gray bars represent the long-standing populations, and the white bars represent the recently-derived populations. The recently-derived populations have all statistically converged to their respective long-standing populations. The A, B, and C-type selection regimes significantly differentiate from each other. The average starvation resistance increased with a longer generation cycle. Statistical analysis was completed with R *lme* and *lsmeans* functions.

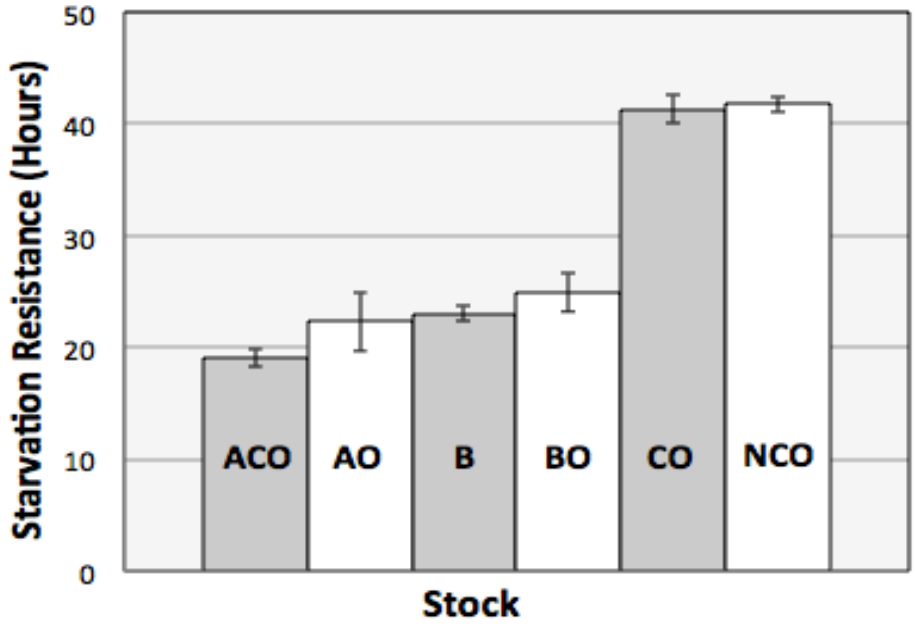

Figure 1.3. Histogram of the average male starvation resistance (hours) for each of the six sets of populations. The gray bars represent the long-standing populations, and the white bars represent the recently-derived populations. The recently-derived populations have all statistically converged to their respective long-standing populations. The C-type selection regimes are significantly different from the A-type and B-type selection regimes. There is a lack of statistical differentiation in male starvation resistance between A-type and B-type populations. The average starvation resistance increased with a longer generation cycle. Statistical analysis was completed with R *lme* and *lsmeans* functions.

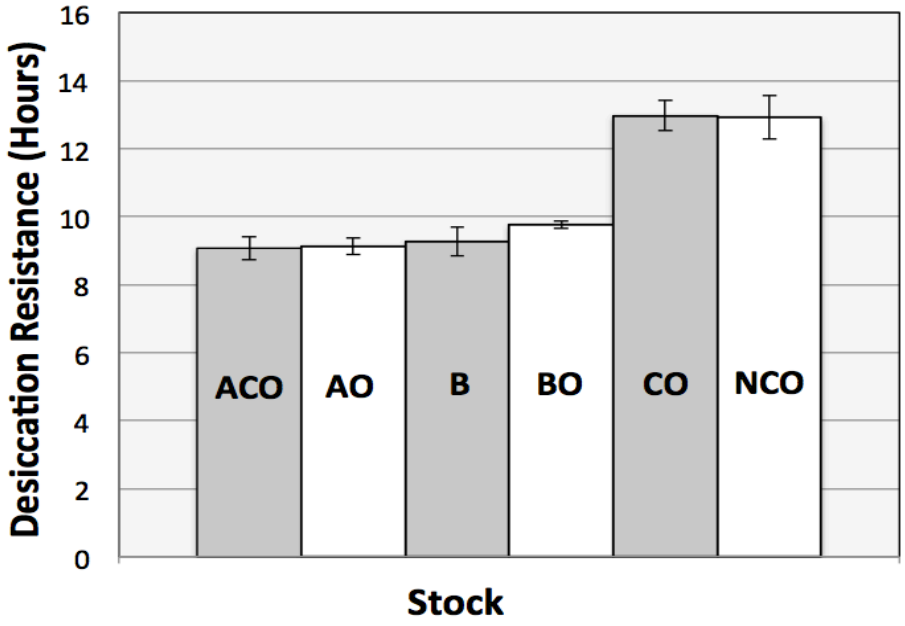

Figure 1.4. Histogram of the average female desiccation resistance (hours) for each of the six sets of populations. The gray bars represent the long-standing populations, and the white bars represent the recently-derived populations. The recently-derived populations have all statistically converged to their respective long-standing populations. The average desiccation resistance is highest in the C-type selection regime, with the A-type and B-type populations not being statistically differentiated from each other. Statistical analysis was completed with R *lme* and *lsmeans* functions.

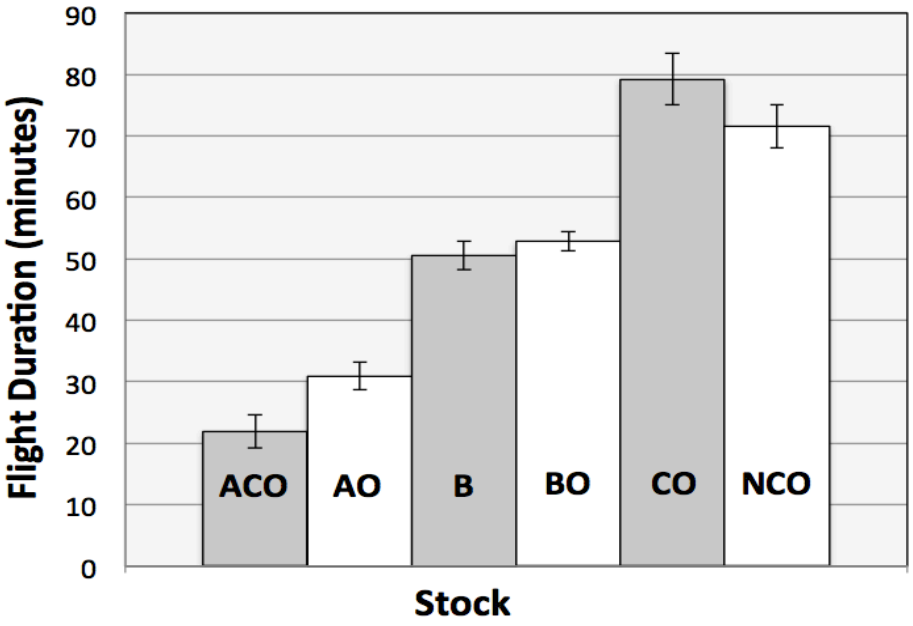

Figure 1.5. Histogram of the average female flight duration time (minutes) for each of the six sets of populations. The gray bars represent the long-standing populations, and the white bars represent the recently-derived populations. The recently-derived B-type and C-type populations have statistically converged to their respective long-standing populations. The recently-derived A-type populations have not converged with their respective long-standing populations. The average flight duration increased with a longer generation cycle. Statistical analysis was completed with R *lme* and *lsmeans* functions.

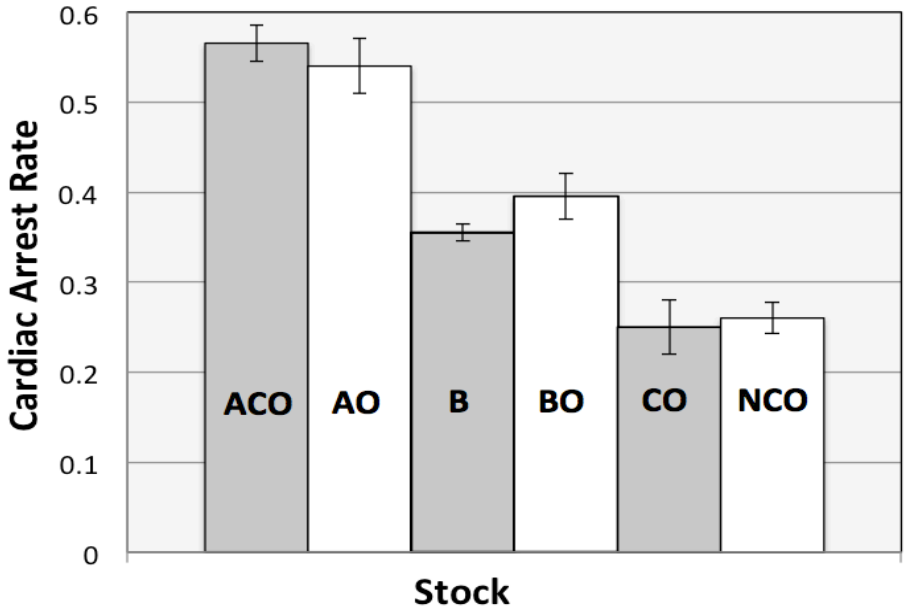

Figure 1.6. Histogram of the average female cardiac arrest rates for each of the six sets of populations after undergoing electrical pacing. The gray bars represent the long-standing populations, and the white bars represent the recently-derived populations. The recently-derived populations have all statistically converged to their respective long-standing populations. Statistical analysis was completed with the Cochran-Mantel-Haenszel test.

CHAPTER 2

Starvation But Not Locomotion Enhances Heart Robustness in *Drosophila*

I. Abstract

Insects and vertebrates have multiple major physiological systems, each species having a circulatory system, a metabolic system, and a respiratory system that enable locomotion and survival in stressful environments, among other functions. Broadening our understanding of the physiology of *Drosophila melanogaster* requires the parsing of interrelationships among such major component physiological systems. By combining electrical pacing and flight exhaustion assays with manipulative conditioning, we have started to unpack the interrelationships between cardiac function, locomotor performance, and other functional characters such as starvation and desiccation resistance. Manipulative sequences incorporating these four physiological characters were applied to five *D. melanogaster* lab populations that share a common origin from the wild and a common history of experimental evolution. While exposure to starvation or desiccation significantly reduced flight duration, exhaustion due to flight only affected subsequent desiccation resistance. A strong association was found between flight duration and desiccation resistance, providing additional support for the hypothesis that these traits depend on glycogen and water content. However, there was negligible impact on rate of cardiac arrests from exhaustion by flight or exposure to desiccant. Brief periods of starvation significantly lowered the rate of cardiac arrest. These results provide suggestive support for the adverse impact of lipids on *Drosophila* heart robustness, a parallel result to those of many comparable studies in human cardiology. Overall, this study underscores clear distinctions among the connections between specific physiological responses to stress and specific types of physiological performance.

II. Introduction

Organisms encounter and endure stress on a daily basis, with most stressors having an adverse impact. However, in animal experiments and patient clinical evaluations, such as cardiac stress tests, stress levels may be increased in order to reveal the physiological foundations of physical health and fitness. Stress resistance, for example, is often an important indicator of components of fitness in lab evolutionary studies that use *Drosophila* (Djawdan et al. 1998; Rose et al. 1992). Stress resistance and energy allocation have been strongly associated with life history and other physiological traits in many *Drosophila* studies of this kind over the years (e.g. Djawdan et al. 1998; Graves and Rose 1990; Graves et al. 1988; Service et al. 1985; Service et al. 1988). Marked differences in the physiological machinery underlying such *Drosophila* characters have been revealed. For example, adult starvation resistance in *Drosophila* depends largely on total stored calories (e.g. Djawdan et al. 1998), while desiccation resistance depends predominantly on water content and rate of water loss (e.g. Gibbs et al. 1997). When Archer et al. (2007) applied sustained strong selection for desiccation resistance in *Drosophila*, water content, and water loss rates were shown to evolve separately from each other.

Understanding responses to stress and stress resistance has been a major theme of invertebrate studies. As previously stated, applying stress can also help define the mechanisms and relationships among different physiological systems. The effects of thermal stress and hypoxia on respiratory and cardiac function have been studied in crustaceans, such as dungeness crab, spiny lobster, or crayfish (Airriess and McMahon 1994; De Wachter and Wilkens 1996; Fitzgibbon et al 2015; McMahon 2001a; McMahon 2001b; McMahon et al. 1974;). Such stressors have also been studied for their effects on metabolite levels in Madagascar cockroaches and in blow flies (Chowanski et al. 2015; Muntzer et al. 2015). For adult mosquitoes, altering

nutrition can have effects on heart contractility, longevity, flight performance, and fecundity (Ellison et al. 2015; Gary and Foster 2001; Kaufmann et al. 2013).

Over the past 100 years, inducing flight has revealed clear relationships between insect locomotor function and metabolic reserves. Dipteran and Hymenopteran species use glycogen, not lipid, as the primary fuel for flight (Clements 1955; Suarez et al. 2005; Vogt et al. 2000; Wigglesworth 1949). Others studies have shown that lipid is the primary flight fuel in Lepidoptera, Hemiptera, and Orthoptera (Amat et al. 2012; Arrese and Soulages, 2010; Beenakkers et al. 1984; Canavoso et al 2003; Ziegler and Schultz 1986; Zera et al. 1999;).

Drosophila cardiac function and its relationship to other physiological processes has been of great scientific interest recently. There are important differences between *Drosophila* and mammalian cardiovascular structure and function. *Drosophila* have an open circulatory system and a reversible direction of hemolymph flow. There is also no relationship between the circulatory system and respiratory system in *Drosophila*. However, there are similarities in early heart development, age-dependent decline in heart function, and the genes associated with heart development, function, and diseases (Bier and Bodmer 2004; Birse and Bodmer 2011; Bodmer and Venkatesh 1998; Cripps and Olson 2002; Diop and Bodmer 2012; Nishimura et al. 2011; Ocorr et al. 2007; Zaffran and Frasch 2002;). In 2004, Bier and Bodmer examined age-dependent decline in cardiac function in *Drosophila* by showing that adult responses to external electrical pacing decline with age. The fruit fly has become a powerful tool for understanding cardiomyopathies, metabolic homeostasis, and other obesity-related disorders (Birse and Bodmer 2011; Diop and Bodmer 2015; Smith et al. 2014; Trinh and Boulianne 2013). *Drosophila* populations exposed to either a high-fat diet or a high-sugar diet have led to flies with cardiac and metabolic dysfunction (i.e. hyperglycemia, insulin resistance, lipid accumulation, reduced

cardiac contractility, and cardiac arrhythmias: Birse et al. 2010; Hoffmann et al. 2013; Na et al. 2013; Trinh and Boulianne 2013).

Here we explore the robustness of physiological interrelationships between flight performance, stress resistance, and cardiac function using experimental manipulation. We conducted electrical pacing assays with manipulative conditioning to determine how cardiac arrest frequency is impacted by flight exhaustion and exposure to desiccation or starvation stresses. Our findings underscore previous findings in some respects, while pointing to the value of combining direct experimental manipulation with other experimental strategies, such as mutation and experimental evolution, in the study of insect physiology.

III. Materials and Methods

3.1 Experimental Overview:

The experimental populations, $B_{1\text{-}5}$, were derived from a single ancestor population, the IV population (Rose 1984; Rose et al. 2004). The IV population originated in 1975 as a sample of *D. melanogaster* caught in Amherst, Massachusetts. After four and a half years of laboratory culture, the $B_{1\text{-}5}$ populations were derived from the single IV population in 1980. The IV and five B populations share a discrete, non-overlapping, two-week generation cycle. These large, outbred populations are maintained on a banana-molasses medium with a 24L:0D light cycle. Seven different manipulative sequences were applied to these $B_{1\text{-}5}$ populations: (i) flight then starvation, (ii) flight then desiccation, (iii) starvation then flight, (iv) desiccation then flight, (v) starvation then pacing, (vi) desiccation then pacing, and (vii) flight then pacing.

3.2 Assay Methods:

Rearing Protocols

Two run-in generations of 14-day life-cycles were used to remove any parental or grand-parental epigenetic effects. The populations were cultured in banana-molasses medium from egg to adult, on a 24L:0D light schedule. Eggs were collected at a density of 60 to 80 eggs per vial after adults were allowed 24 hours to lay eggs. At the end of each run-in generation (day 14 from egg), the populations were transferred to an acrylic cage. Replicate populations of the same number were handled in parallel at all stages. On day 14 of the second run-in generation, the adults were assigned at random to one of the four physiological assays.

Desiccation Resistance Assay

Individual female flies from each population were placed in their own desiccant straw. A piece of cheesecloth separated the fly from the pipet tip at the end of the straw that contained 0.75 grams of desiccant (anhydrous calcium sulfate). The pipette tip containing desiccant was sealed with a layer of Parafilm. Mortality was checked hourly, using lack of movement under provocation as a sign of death. Note that this was a materially different procedure than the one we have employed previously in our studies of desiccation resistance (e.g. Djawdan et al. 1998; Graves et al. 1992; Service et al. 1985), which used vials.

Starvation Resistance Assay

Individual female flies from each population were placed in their own starvation straw with agar. The agar plug provides adequate humidity, but no nutrients. Mortality was checked every four hours, using lack of movement under provocation as a sign of death.

Flight Exhaustion Assay

Female flies from each replicate per stock were first selected at random. The flies were briefly anesthetized using cold-shock by partially submerging a plastic vial with female flies into ice for one to two minutes, and then tethered singly to a monofilament string using Duco cement glue applied to the mesonotum region of the thorax. The flight response was stimulated by gently tapping the tethered string, with total flight duration being recorded. Flight was terminated if the fly could no longer be made to resume flight by tapping within a continuous three-minute interval, or at least seven brief flights were attempted but not sustained consecutively during that period. The protocol for this assay is described further in Graves et al. (1988).

Cardiac Pacing Assay

Female flies from each replicate per stock were first chosen at random. The flies were anesthetized for three minutes using triethylamine, also known as FlyNap, and then placed on a microscope slide prepared with foil and two electrodes. FlyNap was chosen as the anesthetic because of its minimal effect on heart function and heart physiology when administered for more than one minute (Chen and Hillyer 2013). The cold-shock method was not used as an anesthetic for the cardiac pacing assay, because the flies need to be fully anesthetized throughout the procedure. If the flies regain consciousness, the added stress and abdominal contractions while trying to escape would alter heart rate and function more than FlyNap does. Paternostro et al. (2001) found that FlyNap has the least cardiac disruption compared to the two other substances commonly used for *Drosophila* anesthesia, carbon dioxide and ether. Two electrodes were attached to a square-wave stimulator in order to produce electric pacing of heart contraction.

Anesthetized flies were attached to the slide between the foil gaps using a conductive electrode jelly touching the two ends of the fly body, specifically the head and the posterior abdomen tip. The shocking settings for this assay were 40 volts, six Hertz, and 10 ms pulse duration. Each shock lasted for 30 seconds. An initial check of the status of the heart was made after completion of the shock, followed by a check after a two-minute "recovery" period. Heart status was scored as either contracting or in cardiac arrest. The protocol for this assay is outlined in Wessells and Bodmer (2004).

3.3 Manipulative Sequences:

Flight then Starvation

Thirty female flies from each of the five experimental populations underwent the flight exhaustion procedure. An additional 30 flies were tethered, but prevented from flying. If one of these non-flown flies began flying, simply touching the bottom of the legs ended the flight response. These flies were paired with the flown flies. When a flown fly reached exhaustion, the flown fly and the paired non-flown fly were removed from their tethering strings and placed into a straw with agar. The agar plug provides adequate humidity, but no nutrients. To calculate starvation resistance, flies were checked every four hours and time of death was noted if no movement was observed.

Flight then Desiccation

Thirty female flies from each population underwent the flight exhaustion procedure. An additional 30 flies were tethered, but prevented from flying. If one of these designated non-flown flies began flying, simply touching the bottom of the legs ended the flight response. These

flies were paired with the flown flies. When a flown fly reached exhaustion, the flown fly and the paired non-flown fly were removed from the string and placed into a straw with 0.75 grams of desiccant. The desiccant removes moisture, and this environment does not contain any nutrients or water. To record desiccation resistance, flies were checked every four hours and time of death was noted if no movement was observed.

Starvation then Flight

Two hundred female flies from each population were placed into straws with agar. An additional 200 flies were placed into straws with banana-medium. Flies were starved until a 25% mortality threshold was reached (at ~20 hours), and then 30 flies were chosen at random to undergo flight exhaustion. Thirty normally-fed flies were also flown to exhaustion.

Desiccation then Flight

Two hundred female flies from each population were placed into straws with desiccant. An additional 200 flies from each population were placed into straws with banana-medium. Flies were desiccated for 6 hours, which produced approximately 25% mortality, and then 30 flies were chosen at random to undergo flight exhaustion. Thirty normally-fed flies were also flown to exhaustion.

Starvation then Cardiac Pacing

Two hundred female flies from each population were placed into straws with agar. An additional 200 female flies from each population were placed into straws with banana-medium. Flies were starved until a 25% mortality threshold was reached (~20 hours), and then 39-40 flies

per population were chosen at random to undergo cardiac pacing. Forty-41 fed flies per population were also electrically paced.

Desiccation then Cardiac Pacing

Two hundred female flies from each population were placed into straws with desiccant. An additional 200 female flies from each population were placed into straws with banana-medium. Flies were desiccated for 6 hours, which produced approximately 25% mortality, and then 40-45 flies per population were chosen at random to undergo cardiac pacing. Forty-41 fed flies per population were also electrically paced.

Flight then Cardiac Pacing

Fifty female flies from each population underwent the flight exhaustion procedure. An additional 50 flies were tethered, but prevented from flying. These flies were paired with the flown flies. When a flown fly reached exhaustion, the flown fly and the paired non-flown fly were removed from the string and placed into their own respective straw. Within five minutes of reaching exhaustion, both the flown fly and its paired non-flown fly were anesthetized using FlyNap and then electrically paced. Thirty-four to 44 flies per population were electrically paced.

3.4 Statistical Methods:

Linear mixed-effects (LME) models were used to analyze the effect of starvation on flight endurance, the effect of desiccation on flight endurance, the effect of flight exhaustion on starvation resistance, and the effect of flight exhaustion on desiccation resistance. The model for

the effect on starvation on flight duration is described here. The models for the remaining three sequences follow the same format. Let *yijk* be the flight time for treatment – *i* (*i*=1 (control), or 2 (starved)), population – *j* (*j*=1,.., 5) and individual – *k* (*k*=1,.., n_j). We predict flight time with the linear mixed effect model

$$yijk = \alpha + \delta_i\beta + b_j + \varepsilon_{ijk}$$

where δ_i =0, if *i* =1, and 1 otherwise, and b_j and ε_{ijk} are population and individual variation that are assumed to be normally distributed with a zero mean and variance σ_b^2, σ_e^2.

Cochran-Mantel-Haenszel (CMH) tests were used to analyze the rates of cardiac arrests between the "stressed" experimental cohort and the control cohort of flies. The CMH test is used when there are repeated tests of independence, or multiple 2x2 tables of independence. This is the equation for the CMH test statistic, with the continuity correction included, that we used for our statistical analyses:

$$X^2_{\mathrm{MH}} = \frac{\{|\Sigma\left[a_i - \frac{(a_i + b_i)(a_i + c_i)}{n_i}\right]| - 0.5\}^2}{\Sigma(a_i + b_i)(a_i + c_i)(b_i + d_i)(c_i + d_i)/(n_i^3 - n_i^2)}$$

We designated "a" and "b" as the number of cardiac arrests in the stressed and control cohorts of population *i*. We designated "c" and "d" as the number of contracting hearts in the stressed and control cohorts of population *i*. The n_i represents the sum of a_i, b_i, c_i, and d_i. The subscript *i* (i = 1..5), representing one of the five replicate populations within the B stock.

IV. Results

4.1 Effect of Stress on Flight Duration

Experiencing either desiccation or starvation prior to flight negatively impacted the flies' flight endurance (see Figure 2.1 and Figure 2.2). Flies desiccated for a period of six hours flew on average 16.48 minutes, whereas the fed-control flies flew on average 59.69 minutes. The average difference of 43.21 was significantly different when evaluated using the linear mixed effects model (p-value < 0.005). Flies starved for a period of 20 hours flew on average 17.23 minutes, whereas the fed control flies flew on average 54.05 minutes. The average difference of 36.82 minutes was also significantly different when evaluated using the linear mixed effects model (p-value < 0.005). The significant impacts of both starvation and desiccation on flight duration support the notion that surviving these physiological stresses depends on the metabolites that also underlie flight.

4.2 Effect of Flight on Stress Resistance

Prior flight to exhaustion significantly reduces the survival time of flies in a desiccating environment (Figure 2.3). However, flight to exhaustion has a negligible effect on a fly starvation resistance (Figure 2.4). The desiccation survival time of flown cohort of flies was on average one hour less than that of the non-flown tethered cohort of flies. This one-hour difference in survival time was statistically significant when tested using the linear mixed effects model listed above (p-value < 0.01). When starved, flown flies had a slightly longer average survival time. Flown flies survived starvation an average of 2 hours and 18 minutes longer than non-flown flies However, this slight increase in survival time was not statistically significant when tested using the linear mixed effects model given above (p-value = 0.2928). Despite both

starvation and desiccation prior to flight resulting in a lower average flight duration, flight prior to stress resistance only affected desiccation resistance to a degree that reached statistical significance.

4.3 Effect of Stress on Heart Function

When observing the effect of stressors on heart robustness, only one of the three stressors had a statistically significant impact. Flying to exhaustion, or experiencing a brief period of desiccation, does not significantly alter the average cardiac arrest rates of our fruit flies after being electrically paced (Figure 2.5 and Figure 2.6, respectively). The average cardiac arrest rate among the five replicates of flown flies was 27.38%, whereas the average cardiac arrest rate among the five replicates of tethered but not flown flies was 26.26%. The slight difference in cardiac arrests was not statistically significant when evaluated using a Cochran-Mantel-Haenszel test (p-value = 0.933). The average cardiac arrest rate among the five desiccated replicates was 35.44%, whereas the average cardiac arrest rate among the five control replicates was 35.63%. The small increase of cardiac arrest rate in the control flies was not statistically significant when evaluated using a Cochran-Mantel-Haenszel test (p-value = 0.951).

Unlike the neutral effects of flight or desiccation on cardiac arrest rates, there was a decrease in the average cardiac arrest rate in flies exposed to starvation prior to electrical pacing (Figure 2.7). The average cardiac arrest rate among the five replicates of starved flies was 19.5%, whereas the average cardiac arrest rate among the five replicates of control fed flies was 36%. The difference in cardiac arrests rates between the five starved and five control groups was statistically significant when evaluated using the Cochran-Mantel-Haenzsel test (p-value < 0.001). Starving a cohort of flies for a period of 20 hours appears to lower the average cardiac

arrest rate. The same cannot be said for the six-hour exposure to a desiccated environment, or being flown to exhaustion. Thus we find an association only between rates of cardiac arrest and prior starvation.

V. Discussion

5.1 Physiological Interrelationships of Stress Resistance and Flight Performance

Exposure to starvation or desiccation significantly reduced flight duration. The results from these two manipulative sequences strengthened the hypothesis that stress of any kind for a prolonged period of time will weaken the body and thereby undermine at least some functions. These results supported what we initially expected, a diminished flight duration after imposition of either of these two environmental stressors. However, the only definitive conclusion we can make is that these two stressors affect flight duration. These two manipulative procedures only indicated one direction of causation, and accordingly we sought to characterize the converse pattern(s) of causation by testing for the effect of flight prior to stress resistance.

The experiments testing the effect of flight on stress resistance found that flight exhaustion significantly affected survival time under desiccation, but did not affect survival time under starvation. The lack of an adverse effect of flight on starvation resistance supports the hypothesis that *Drosophila* flight performance is not dependent on lipid content, a long-established conclusion about flight in dipteran species (Clements 1955; Wigglesworth 1949; Williams et al. 1943). Our manipulative results indirectly corroborate what previous evolutionary physiology research has shown, specifically that (1) glycogen and trehalose are the major sources of energy for insect flight (Graves et al. 1992), and (2) there is a significant relationship between desiccation and glycogen, trehalose, and bulk water content (Archer et al.

2007; Gibbs et al. 1997). Trehalose and glycogen, which serve as the primary fuel reserves for flight, are more readily used by fly flight musculature than lipids (Graves et al. 1992; Nation 2008). Despite the impact of starvation on flight endurance, our finding of minimal impact of flight on starvation resistance supports the common conclusion that there is little relationship between flight performance and lipid content in Diptera, as the most important determinant of starvation resistance in *Drosophila* is lipid content (vid. Djawdan et al., 1998). That is, these results are readily incorporated into previous accounts of the distinct roles of glycogen and lipid as substrates for specific physiological functions.

5.2 The Response of Cardiac Robustness to Various Stressors

We found that only one of three prior stressors significantly altered cardiac arrest rates, and in a direction we did not initially expect. Flight exhaustion or exposure to desiccation did not alter the rate of cardiac arrests in fruit flies. But starving a population of fruit flies for a period of 20 hours significantly lowered the rate of cardiac arrests due to electrical pacing. The negligible effects of flight exhaustion and desiccant exposure prior to electrical pacing suggests that glycogen content and water loss do not affect the rate of cardiac arrest. That fruit flies subjected to starvation stress can have a *reduced* rate of cardiac arrests when electrically paced (Figure 2.7) suggests that there is an important adverse effect of body fat on heart function in fruit flies, a parallel result to those of many comparable studies in human cardiology (Crewe et al., 2013; Heinrichsen and Haddad, 2012; Manrique et al., 2013).

5.3 A Divide Among the Physiological Interrelationships

From the effects of these seven manipulative sequences, we can infer an overall view of interrelationships among several major physiological systems in *D. melanogaster* (see Figure 2.8). A significant impact was seen in four of the seven manipulative sequences. In certain manipulative sequences, one of the replicated populations may display a neutral response to the stressor, or a response opposite to the overall observed trend. Even though the observed overall trend may not be universal among all five replicated populations in each manipulative sequence, we can still find an overall trend moving in one direction or another when using all five replicated populations. As proposed in our earlier work (e.g. Graves et al. 1992), we continue to find a clear divide not only between certain physiological processes, but also between the metabolic reserves used in physiological responses to particular stressors and specific types of physiological performance. On one side, there is evidence of strong relationships among desiccation resistance, flight exhaustion, and carbohydrate content, once again corroborating the findings of past studies (e.g. Archer et al. 2007; Gibbs et al. 1997; Graves et al. 1992; Wigglesworth 1949). And on the other side, we see a strong relationship among starvation resistance, cardiac robustness, and lipid content (Figure 2.8). These results suggest that lipid content, not carbohydrate content, is a key determinant of cardiac robustness and a fly's ability to resist cardiac arrest. However, our interpretations of the biochemical underpinnings of the effects we have found cannot be considered definitive. Instead, our results are no more than suggestive of the biochemical hypotheses we have offered.

With a high metabolic rate being typical in *Drosophila*, we hypothesize that flies undergoing starvation consume a significant amount of free-floating lipids in their hemolymph. The improved cardiac robustness after a fasting period is similar to the results found by Hardy et

al (2015). Fasting their starvation-selected, laboratory-evolved populations for seven days rescued heart function (Hardy et al. 2015). One possible mechanism behind the disruptive effect of fat on heart function is the mechanical effect of enlarged fat body structures applying pressure on the heart anatomically (Hardy et al. 2015). Another potential effect of a moderate period of starvation could be reduced hemolymph viscosity, which may then make fly heart contraction less demanding physiologically during cardiac pacing. To determine whether the hemolymph is in fact less viscous after starving for 20 hours, future experiments should examine the lipid content in the hemolymph and fat bodies both before and after experiencing periods of starvation, among other measures of heart mechanics with and without starvation.

5.4 Understanding Invertebrate and Vertebrate Physiology and Disease

Experimentally probing these physiological interrelationships in *D. melanogaster* has improved our understanding of this complex metazoan as an experimental model for the study of health and disease. *Drosophila* has been a key model organism for many fields, including experimental evolution and physiology (e.g. Burke and Rose 2009). *Drosophila* have short generation times, are easily maintained at large population sizes, and possess vast public genomic resources (e.g. FlyBase). Unlike most microbial models, the physiology of *Drosophila* is both complex and broadly analogous to some features of vertebrate physiology. *D. melanogaster* also have orthologous genetic mechanisms with those that are thought to determine lifespan among vertebrates, including such genetic systems as TOR and insulin/insulin-like signaling (e.g. Partridge and Gems 2007).

With cardiovascular disease and heart-related defects being leading causes of death among Western patients, conducting useful heart experiments at great scale and intensity with

model organisms should be of significant value (Olson 2004). As mentioned earlier, fruit flies experience a decline in heart function and robustness with age, similar to what has been found in aging human adults (Bier and Bodmer 2004; Nishimura et al. 2011; Ocorr et al. 2007; Paternostro et al. 2001; Wessells and Bodmer 2007). Here we found that flies with higher lipid content have decreased heart robustness. Likewise, it is believed that higher circulating levels of triglycerides in humans are a major factor affecting cardiac disease. The *Drosophila* fly shares some of the genes that underlie its cardiac performance with those of human cardiac genetics, such as *tinman* (Bodmer 1993; Bodmer 2006; Bodmer and Venkatesh 1998) and *opa1* (Shahrestani et al. 2009). In another study of *Drosophila* cardiac function, Birse et al. (2010) have shown that altering nutrient-sensing signaling pathways (e.g. insulin-TOR signaling) can combat the adverse cardiac effects of a high-fat diet. Additional genes and signaling pathways conserved between *Drosophila* and mammals might be used to develop therapies that could counteract the lipotoxicity and cardiac dysfunction of obesity (e.g. PGC-1/spargel: Diop et al. 2015). The combination of the present study's results with the results of other studies, such as Birse et al. (2010), Diop and Bodmer (2012), Diop et al (2015) or Hardy et al. (2015), underscores the value of using laboratory-evolved *Drosophila* populations to parse the genetic and physiological mechanisms of heart function.

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VIII. Figures and Figure Legends

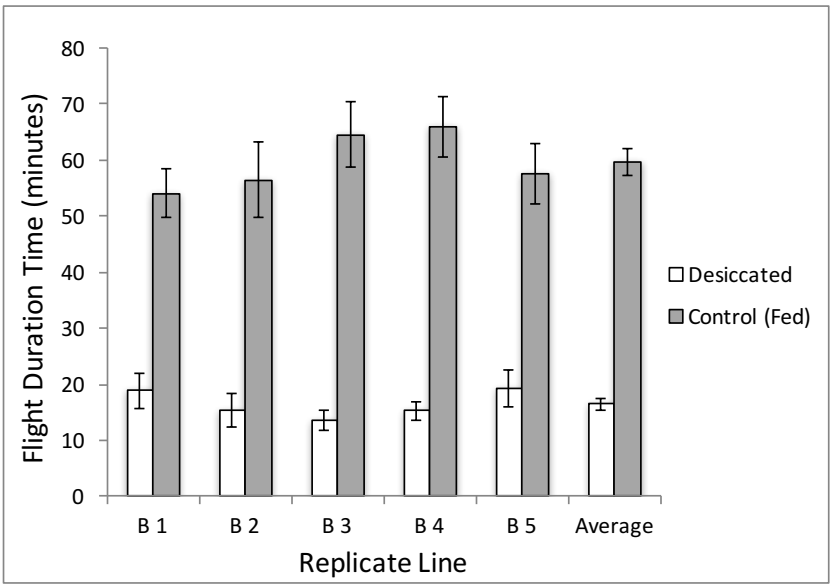

Figure 2.1. The average flight duration of flies desiccated for a brief period of time compared to the flies fed the standard banana-medium (mean ± 1 SEM). Desiccated flies had an average flight duration significantly lower than the control fed flies (p-value < 0.005).

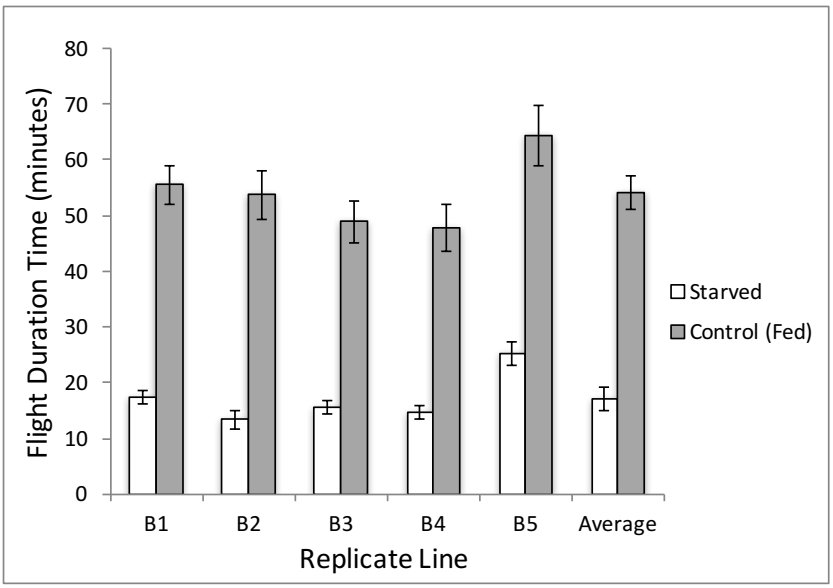

Figure 2.2. The average flight duration of starved fruit flies compared to the average flight duration of fruit flies fed the standard banana-medium (mean ± 1 SEM). Starved flies had an average flight duration significantly lower than the control flies (p-value < 0.005).

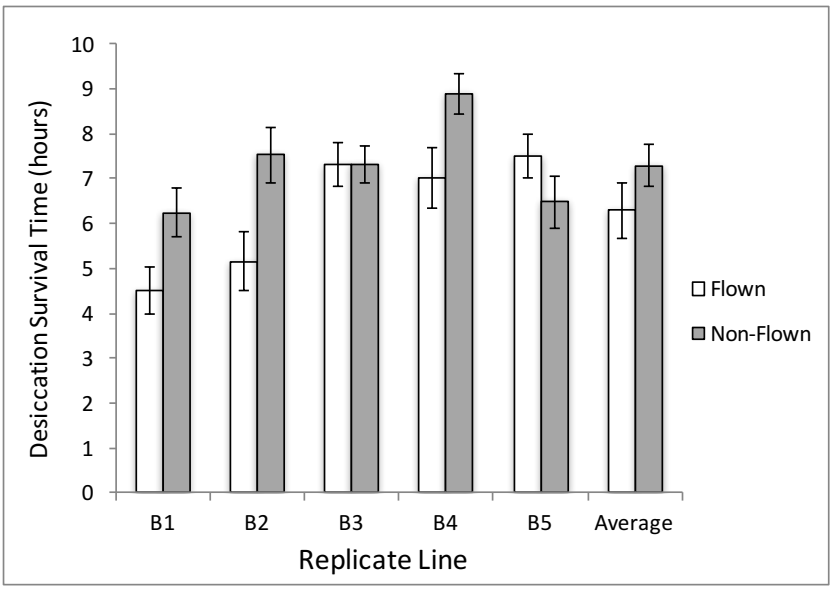

Figure 2.3. The average survival time of fruit flies in a desiccated environment after being flown to exhaustion (mean ± 1 SEM). The non-flown cohort was tethered, but prevented from flying. Exhausted flies had an average desiccation survival time significantly lower than the non-exhausted flies (p-value < 0.0055).

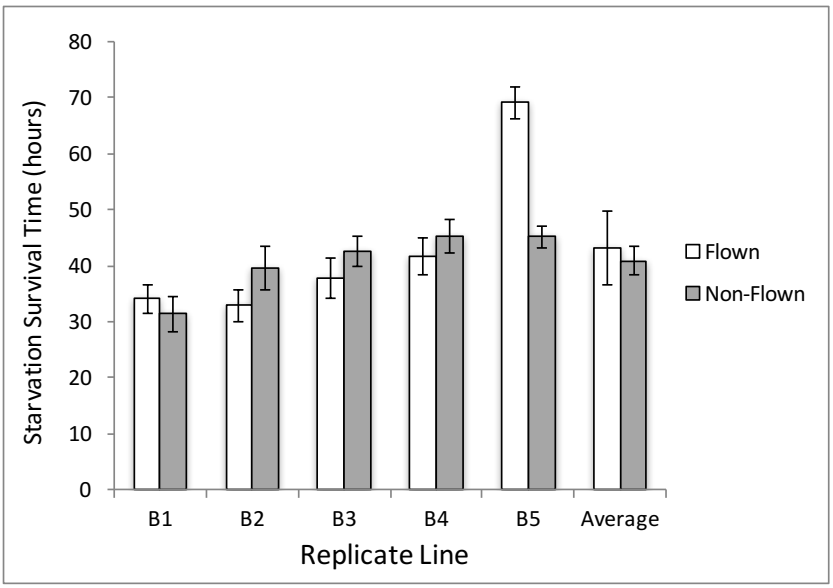

Figure 2.4. The average survival time of fruit flies in a starvation environment after being flown to exhaustion (mean ± 1 SEM). The non-flown cohort was tethered, but prevented from flying. Exhausted flies had an average starvation survival time that was not significantly different than the non-exhausted flies (p-value < 0.2928).

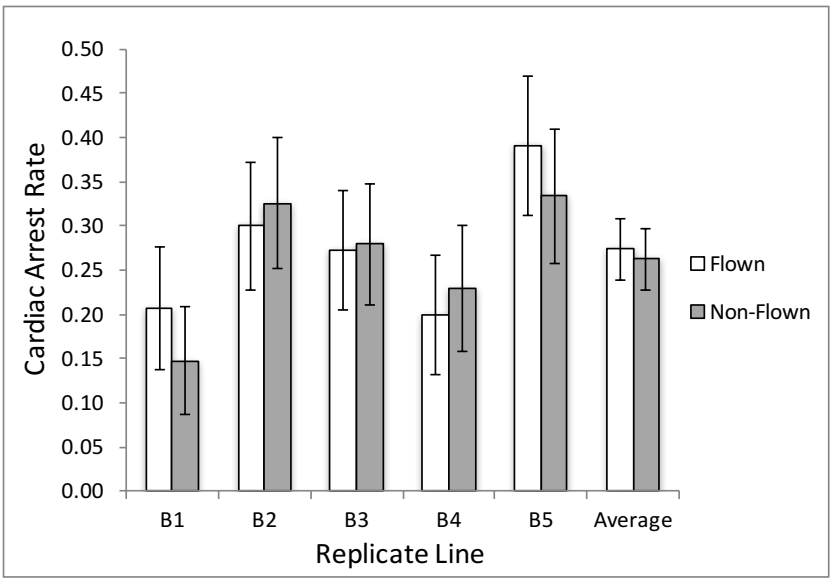

Figure 2.5. The average rate of cardiac arrest of fruit flies after being flown to exhaustion, compared to flies that were tethered, but prevented from flying (mean ± 1 SEM). Exhausted flies had an average rate of cardiac arrests that was not significantly different than the non-exhausted flies (p-value = 0.933).

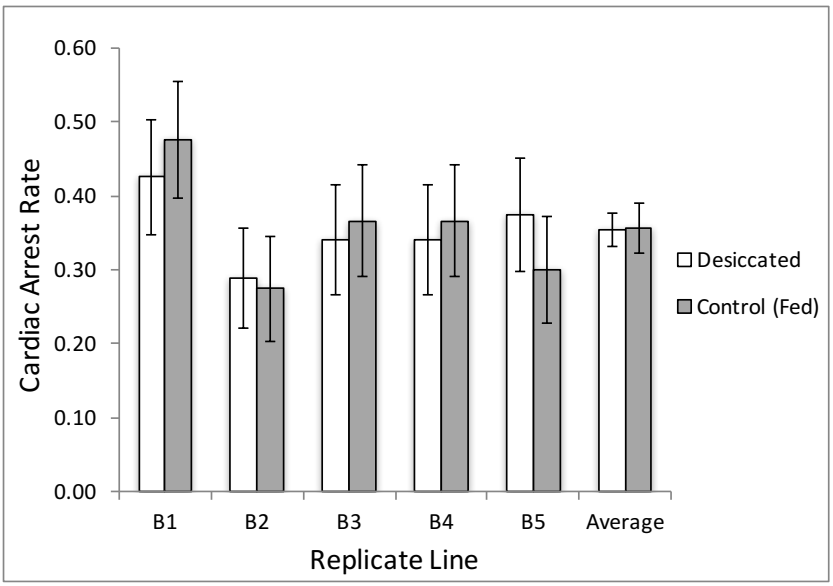

Figure 2.6. The average rate of cardiac arrests in desiccated fruit flies compared to flies that were fed the standard banana-medium (mean ± 1 SEM). Desiccated flies had an average rate of cardiac arrests that was not significantly different than the control fed flies (p-value = 0.951).

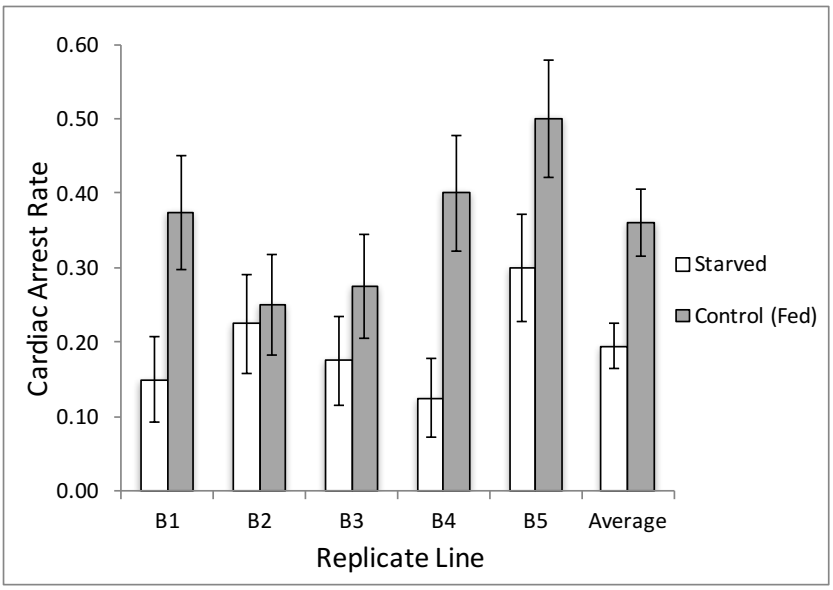

Figure 2.7. The average rate of cardiac arrests in starved fruit flies compared to flies that were fed the standard banana-medium (mean ± 1 SEM). Starved flies had an average rate of cardiac arrests that was significantly lower than the control fed flies (p-value < 0.001).

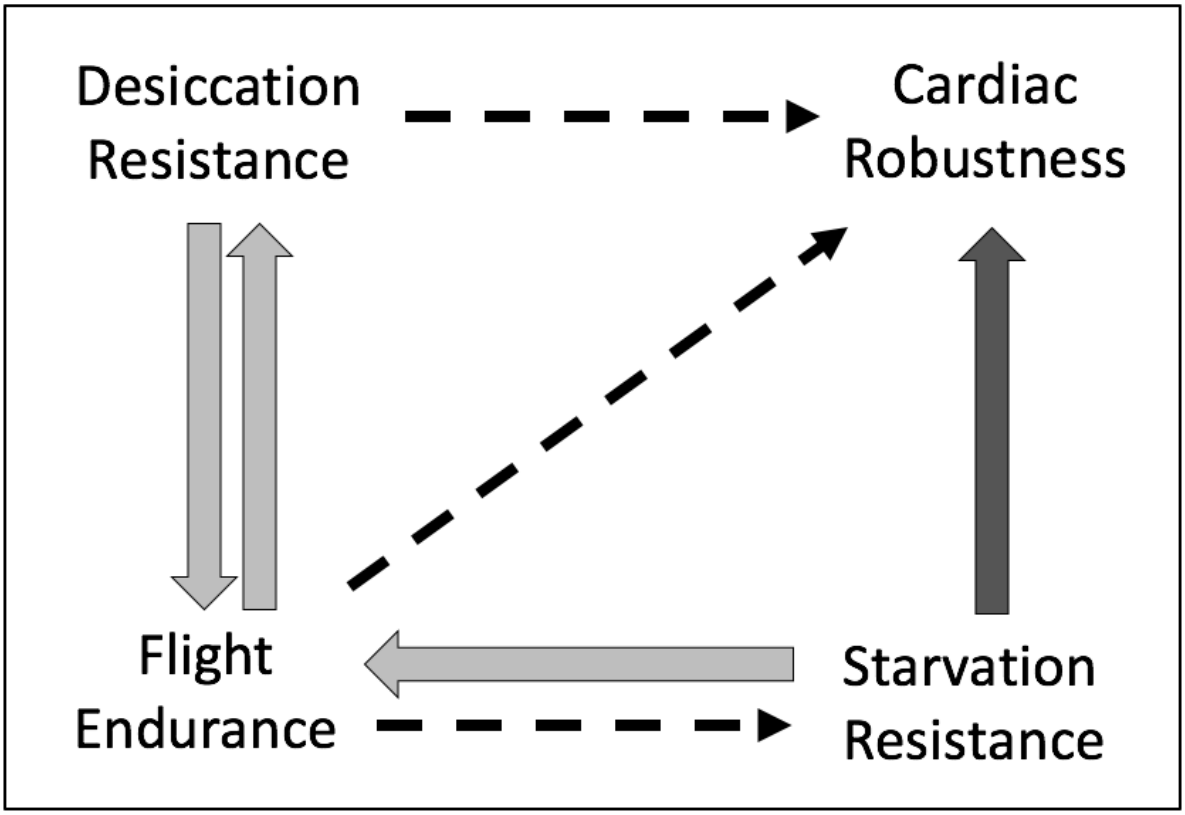

Figure 2.8. The physiological interrelationships between *Drosophila* flight performance, stress resistance, and cardiac robustness. This is a visual summary of the seven manipulative sequences applied to the $B_{1\text{-}5}$ populations. A black, dotted arrow indicates a neutral effect of a pre-lethal stress on a physiological process (i.e. exposure to desiccation on cardiac arrest rates). A light grey, solid arrow indicates a negative effect of a pre-lethal stress on a physiological process (i.e. flying to exhaustion on desiccation survival time). A dark grey, solid arrow indicates a positive effect of a pre-lethal stress on a physiological process (i.e. exposure to starvation on cardiac arrest rates).

CHAPTER 3

The Effects of Intense Selection for Starvation Resistance on *Drosophila* Physiology

I. Abstract

In experimental evolution, we impose functional demands on laboratory populations of model organisms using selection. After enough generations of such selection, the resulting populations constitute excellent material for physiological research. In effect, evolutionary physiologists are often able to produce physiologically differentiated *Drosophila* populations at will. An intense selection regime for increased starvation resistance was imposed on ten, large, outbred *Drosophila* populations. We observed the response of starvation and desiccation resistance (generation 55), metabolic reserves (generation 67), and heart robustness via electrical pacing (generation 62). As expected, significant increases in starvation resistance and lipid content were found in our ten intensely-selected SCO populations. The selection regime also indirectly improved desiccation resistance, water content, and glycogen content among these populations. Additionally, the rate of cardiac arrests in our ten obese SCO populations was doubled the rate in the ten ancestral CO populations. Finally, age-specific mortality rates were increased at early adult ages by selection. The cardiac dysfunction, increased adult mortality, and elevated lipid levels in the SCO populations make them a useful model system for heart disease and obesity-related disorders.

II. Introduction

Organisms encounter varied stresses such as those that arise from reproduction, food deprivation, predation, and temperature fluctuations. The life span of an individual is partly

determined by the frequency of its periods of stress, the severity of such stress, and its ability to cope with the stresses to which it is exposed (Vermeulen and Loeschcke 2007). Natural selection sometimes produces adaptive stress responses that increase an organism's ability to cope to a stressful situation. Trade-offs with life history characters often shape or constrain the evolution of such adaptive stress responses (Nesse and Young 2000).

Stress affects both vertebrate and invertebrate organisms throughout their lifetime, and both groups experience and defend against similar stressors, sometimes but not always using the same or similar physiological machinery. For example, caloric and other forms of dietary restriction have been useful manipulations for probing mechanisms of ageing in yeast, nematodes, fruit flies, spiders, rodents, rhesus monkeys and humans (Anderson et al. 2003; Austad 1989; Fontana et al. 2004; Houthoofd et al. 2005; Jiang et al. 2000; Lane et al. 2002; Masoro 1998; Masoro 2005; Partridge et al. 2005). The effects of thermal stress and hypoxia on metabolic levels, respiratory function, and cardiac function have been studied in cockroaches, blow flies, fruit flies, crustraceans, and rodents (Airrieses and McMahon 1994; Azad et al. 2009; Cai et al. 2003; Chowanski et al. 2015; De Wachter and Wilkens 1996; Fitzgibbon et al 2015; Haddad and Donnelly 1990; McMahon 2001a; McMahon 2001b; Muntzer et al. 2015).

Experimental evolutionists take advantage of stress, using it as a tool either (1) to measure an organism's physical robustness, or (2) to create differentiated populations with which to study adaptation. In the latter case, such populations provide excellent material for physiological analysis of organismal functions related to stress. Adapting to stress can create life-historical advantages (e.g. increased longevity) and disadvantages (e.g. lower fecundity) that may also be of interest in studies of experimental evolution. Selection for increased resistance to starvation or desiccation in *Drosophila* can result in slower development, reduced larval or pupal

viability, reduced early fecundity, and increased adult longevity (Chippindale et al. 1996; Rose 1984; Rose et al. 1990; Rose et al. 1992). Temperature, a complex variable that can affect respiration, growth, and reproduction, has been the focus of many experimental evolution experiments in *Escherichia coli* (Bennett et al. 1990; Cooper et al. 2001; Mongold et al. 1999; Tenaillon et al. 2012). For example, Brandon Gaut and his colleagues at UC Irvine selected for thermal tolerance in more than 100 replicate populations of *E. coli* for more than 2000 generations (Tenaillon et al. 2012). In their selection experiment, Tenaillon et al. (2012) found two major genetic pathways, *rpoBC* and *rho*, involved in their populations' adaptation to thermal tolerance.

A common model system for experimental evolution has been the complex metazoan model genus, *Drosophila*. Rapid physiological changes often arise quickly in response to selection in *Drosophila* populations, whether a specific physiological characters has been targeted for selection or not (Burke and Rose 2009). The short generation-time and ease of maintenance of *Drosophila* populations, their available genomic data, as well as prior physiological research with the genus make the fruit fly a model system which is especially suited to studies of evolutionary physiology. Thus the last 30 years have seen appreciable progress in the study of the evolutionary physiology of *Drosophila* species, especially *D. melanogaster.*

A common theme of *Drosophila* evolutionary physiology has been the study of the physiological basis for the experimental evolution of populations that have been selected for very different life-histories (reviewed in Rose et al. 2004). It is important to note that, though populations with different life-cycle timings are not specifically selected for different levels of stress resistance, they do nonetheless show distinctive enhancements in a variety of stress and

performance characters. For example, early on it was found that a variety of stress resistances and performance characters were improved in experimentally evolved stocks with postponed senescence (Graves and Rose 1990; Graves et al. 1992; Rose et al. 2004; vid. Service et al. 1985). Service et al. (1985) first analyzed the effects of postponed senescence on stress resistance. Both male and female flies displayed an increase in starvation resistance, desiccation resistance, and resistance to ethanol vapor (15%). In addition to desiccation resistance, Graves et al. (1988) as well as Graves and Rose (1990) showed that selection for postponed senescence also increased flight endurance. Physiological analysis of stress resistance in populations cultured with different life cycles has revealed evolutionary changes in multiple physiological characters, from lipid and glycogen content to water content and rates of water loss (Graves et al. 1992; Chippindale et al. 1998; Djawdan et al. 1998; Gibbs et al. 1997; Rose et al. 2004). Djawdan et al. (1998) found strong, positive correlations between energy content and starvation resistance in both male and female flies. However, only carbohydrate content in female flies was positively correlated with desiccation resistance, indicating desiccation resistance was a more complex character. Selection for moderate increases in these stress resistance capacities also increased mean life span (Rose et al. 1992), but this effect is reversed at still greater levels of stress resistance (Archer et al. 2003; Archer et al. 2007; Phelan et al. 2003).

Our lab and our colleagues have gone on to examine the response of life history and physiological traits to intense, or focal, selection regimes (e.g. selection for increased starvation resistance), moving away from studies that looked at the evolutionary effects of particular laboratory life-cycle selection regimes. We have called this type of selection paradigm "culling selection" in the past (e.g. Rose et al. 1990), but it might be better described as *intense selection.* An intense selection regime can eventually achieve extreme levels of functional differentiation.

Such extreme functional differentiation is achieved by the use of environments so inimical to survival that only a small percentage of each generation survives selection. Our past studies have shown that such stringently selected populations are useful material for analyzing the mechanistic foundations of adaptation in lab fruit flies. For example, Archer et al. (2007) produced flies that can survive complete desiccation for as much as ten times longer than unselected flies. And from these populations, they were able to provide further insight into the relationship among the mechanisms underlying desiccation resistance. Archer et al. (2007) were able to show that two of the characters that underpin desiccation resistance, higher water content and lower water loss rates, evolve with relative independence from each other. Thus experimental evolution is an important tool for parsing the interconnections between the physiological machinery underlying life-history characters (Archer et al. 2007; Bradley et al. 1999; Burke and Rose 2009; Graves et al. 1992).

Here we present a well-replicated set of populations intensely selected for increased starvation resistance: ten "SCO" populations that have been undergoing selection since August 2010. This intense selection regime rapidly produced changes in body shape, body size, and stress resistance. We knew from previous experiments employing a similar selection regime for starvation resistance that this selection regime would increase both starvation resistance and lipid content (Djawdan et al. 1998; Harshmann et al. 1999; Rose et al. 1992). "Pseudocomparative" studies (vid. Burke and Rose, 2009) and reverse-selection studies (e.g. Passananti et al. 2004) have already found positive correlations among starvation resistance, lipid content, and longevity. But other studies have shown that intense selection regimes, which push functional characters to extreme levels, can lead to a breakdown in these correlations (Archer et al. 2003; Phelan et al. 2003).

For this study, we functionally characterize our ten, intensely selected, starvation resistant populations. In particular, we compare them to both their ancestors, which have not been subjected to such intense focal selection, as well as five other populations that have converged on their ancestors with respect to both life history (Burke et al. 2016) and genomics (Graves et al. 2017). We examine functional characters that have long been studied in *Drosophila*, specifically stress resistance, metabolic reserves and longevity, as well as a functional character of more recent interest in our laboratory, heart robustness. The present study shows how rapid and intense selection can induce extensive functional differentiation, and its potential as a tool for obtaining new insights into 1) the physiological foundations of adaptation and 2) heart disease and other obesity-related disorders.

III. Materials and Methods

3.1 Populations Used

This study employed 20 of the large, outbred, and highly differentiated populations created by the Rose laboratory over last 37 years (Rose 1984; Rose et al. 1992; Rose et al. 2004). All of the populations assayed here descend from a single *Drosophila melanogaster* population, called IV. The IV population originated in 1975 as a sample of *D. melanogaster* caught in Amherst, Massachusetts. After four and a half years of laboratory culture, the B_{1-5} populations (baseline) and O_{1-5} populations (70-day generation cycle) were derived from the single IV population in 1980 (Rose 1984; Rose et al. 2004). The 20 populations studied here are referred to as CO_{1-5}, nCO_{1-5}, SCO-a_{1-5}, and SCO-b_{1-5}.

The CO_{1-5} populations (28-day generation cycle) were derived from the O_{1-5} populations in 1989 (Rose et al. 1992). The nCO_{1-5} populations (28-day generation cycle) were derived from

the $O_{1\text{-}5}$ populations in 2009. The $nCO_{1\text{-}5}$ populations are kept under identical conditions as those given the $CO_{1\text{-}5}$ populations (Burke et al. 2016). This culture regime is referred to as the C-type treatment.

The SCO-$a_{1\text{-}5}$ and SCO-$b_{1\text{-}5}$ populations (28-day generation cycle) are ten populations intensely selected for starvation resistance. These populations were derived from the $CO_{1\text{-}5}$ populations in August 2010, using the following "HSH" selection protocols, first published in Phelan et al. (2003). There are two five-fold sets of CO populations called "alpha" and "beta", which are regularly crossed within-replicates (e.g. CO1-a and CO1-b). SCO-$a_{1\text{-}5}$ were derived from the alpha set $CO_{1\text{-}5}$ populations, and SCO-$b_{1\text{-}5}$ were derived from the beta set of $CO_{1\text{-}5}$ populations. After a two-week development period, the flies are fed a high-yeast diet for three days before receiving a nonnutritive agar during the starvation period. Each population is exposed to the nonnutritive agar until a 75-80% mortality threshold has been reached. A three-day high yeast diet period follows the starvation period with egg collection for the succeeding generation occurring on day 28. At the beginning of this experiment, the starvation period took just three days to achieve 75-80% mortality. Currently, the starvation period lasts for approximately 10 days. At the time of the experimental assays reported here, the SCO populations had experienced up to 67 generations of intense selection. The group of populations subjected to this selection regime is referred to as the S-type treatment group.

These large, outbred populations are maintained on a banana-molasses medium with a 24L:0D light cycle. Each new population was founded from the same number replicate of their ancestral population. All 20 populations were kept at moderately large census populations sizes ($N > 1{,}000$) to avoid confounding inbreeding effects. All 20 populations experience a 14-day

developmental period in vials before being transferred to an acrylic cage for the remainder of their generation cycle.

3.2 Assay Methods

Rearing protocols

Two run-in generations of 14-day life-cycles were used to remove any parental or grand-parental epigenetic effects. The populations were cultured in banana-molasses medium from egg to adult, on a 24L:0D light schedule. Eggs were collected at a density of 60 to 80 eggs per vial after adults were allowed 24 hours to lay eggs. At the end of each run-in generation (day 14 from egg), the populations were transferred to an acrylic cage. Replicate populations of the same number were handled in parallel at all stages. On day 15 of the second run-in generation, the adults were assigned at random to one of the following eight assays.

Glycogen Content

For each population, six groups of 10 females were anesthetized using ethyl ether and individually placed in 1.7 milliliter microcentrifuge tubes. The next day, each group was placed in aluminum weighing boats and placed in an oven for one hour at 60 ºC. Each group was transferred to their respective microcentrifuge tube where 700 microliters was added to each tube. The flies were then grounded using a hand-held battery-operated grinder. Each tube was boiled in water for five minutes. Once complete, 100 microliters from each tube was transferred to a 13 x 100 millimeter test tube. Three milliliters of an anthrone reagent was added to each test tube. The anthrone reagent composition was 150 milligrams of anthrone per 100 milliliters of 72% sulfuric acid. Each test tube was then incubated in a water bath set to 90 ºC for 10 minutes.

Two one-milliliter samples from each test tube were placed in their own cuvettes. Absorbance was then measured using a Perkin Elmer Lambda spectrophotometer at a wavelength of 620 nanometers. All measurements were taken within 10 minutes of being removed from the water bath. Five controls of known glycogen concentration underwent the same process. At the beginning of this assay, the CO and nCO populations have been under 352 and 98 generations of C-type selection. The SCO-a and SCO-b populations completed 67 generations of intense selection.

Water Content

For each population, six groups of 10 females were anesthetized using ethyl ether. Each group was placed in aluminum weighing boats, and had their wet mass measured. The flies were frozen over night, and placed in a drying oven the next day at 60 °C for 24 hours. After the 24 hours, each group was reweighed. The difference between the wet mass and dry mass represented the water content of that group. At the beginning of this assay, the CO and nCO populations have been under 352 and 98 generations of C-type selection. The SCO-a and SCO-b populations completed 67 generations of intense selection.

Lipid Content

For each population, lipid content was measured for six groups of 10 females. After the dry masses of the groups were recorded for the water content assay, each group was placed in their own Whatman thimble. The thimbles were placed in the extractor of a Soxhlet apparatus. Petroleum ether was used as the extraction solvent. The thimbles were in the Soxhlet apparatus for 24 hours, and upon completion, were removed and placed in a drying oven at 60 °C for one

hour. The post-extraction mass of each group was recorded using a microbalance scale. The difference in the dry mass and the post-extraction mass of each group represented the specific group's lipid content. At the beginning of this assay, the CO and nCO populations have been under 352 and 98 generations of C-type selection. The SCO-a and SCO-b populations completed 67 generations of intense selection.

A second set of lipid content measurements were made for the CO_{1-5} and SCO-a_{1-5} at six different adult ages. These measurements conducted at ages 14, 21, 28, 35, 42, and 49 days from egg. The CO populations have been under C-type selection for 357 generations at time of egg collection. The SCO-a populations completed 86 generations of selection at time of egg collection. The protocol is the same as above.

Desiccation Resistance Assay

Thirty individual female flies from each population were placed in their own desiccant straw. A piece of cheesecloth separated the fly from the pipet tip at the end of the straw that contained 0.75 grams of desiccant (anhydrous calcium sulfate). The pipette tip containing desiccant was sealed with a layer of Parafilm. Mortality was checked hourly, using lack of movement under provocation as a sign of death. Note that this was a materially different procedure than the one we have employed previously in our studies of desiccation resistance (e.g. Djawdan et al. 1998; Graves et al. 1992; Service et al. 1985), which used vials. At the beginning of this assay, the CO and nCO populations have been under 325 and 72 generations of C-type selection. The SCO-a and SCO-b populations had completed 55 generations of intense selection.

Starvation Resistance Assay

Thirty individual female flies from each population were placed in their own starvation straw with agar. The agar plug provides adequate humidity, but no nutrients. Mortality was checked every four hours, using lack of movement under provocation as a sign of death. At the beginning of this assay, the CO and nCO populations have been under 325 and 72 generations of C-type selection. The SCO-a and SCO-b populations had completed 55 generations of intense selection.

Cardiac Pacing Assay

Forty female flies from each replicate per stock were first chosen at random. The flies were anesthetized for three minutes using triethylamine, also known as FlyNap, and then placed on a microscope slide prepared with foil and two electrodes. FlyNap was chosen as the anesthetic because of its' minimal effect on heart function and heart physiology when administered for more than one minute, but less than the lethal time of five minutes (Chen and Hillyer 2013). The cold-shock method was not used as an anesthetic for the cardiac pacing assay, because the flies need to be fully anesthetized throughout the procedure. If the flies regain consciousness, the added stress and abdominal contractions while trying to escape would alter heart rate and function more than the side-effects of FlyNap's do. Paternostro et al. (2001) found that FlyNap has the least amount of cardiac disruption compared to the two other substances commonly used for *Drosophila* anesthesia, carbon dioxide and ether. Two electrodes were attached to a square-wave stimulator in order to produce electric pacing of heart contraction. Anesthetized flies were attached to the slide between the foil gaps using a conductive electrode jelly touching the two ends of the fly body, specifically the head and the posterior abdomen tip. The shocking settings

for this assay were 40 volts, six Hertz, and 10 ms pulse duration. Each shock lasted for 30 seconds. An initial check of the status of the heart was made after completion of the shock, followed by a check after a two-minute "recovery" period. Heart status was scored as either contracting or in cardiac arrest. The protocol for this assay is outlined in Wessells and Bodmer (2004). At the beginning of this assay, the CO and nCO populations were under C-type selection for 332 and 79 generations, respectively. The SCO-a and SCO-b populations completed 62 generations of intense selection.

A second set of cardiac arrest rate measurements were made for the $CO_{1\text{-}5}$ and SCO-$a_{1\text{-}5}$ at six different adult ages. These measurements conducted at ages 14, 21, 28, 35, 42, and 49 days from egg. The CO populations have been under C-type selection for 357 generations at time of egg collection. The SCO-a populations completed 86 generations of selection at time of egg collection. The protocol is the same as above.

Adult Mortality

Flies from all populations of the CO, nCO, and SCO selection treatments were handled in parallel for two run-in generations in vials. After 14 days of development in vials, approximately 800 to 1200 adult flies from each of the populations of the CO, nCO, SCO-a, and SCO-b were transferred into Plexiglass cages, with multiple cohort cages for each population. From then on they were fed a standard banana-molasses diet with yeast, the food being replaced every day. Dead flies were collected from cages and counted at the same time every day. Mortality data were obtained over all adult ages for the CO, nCO, SCO-a, and SCO-b populations. Each assayed cohort was initially maintained in five cages; to control population density over time, cages were consolidated as the number individuals in a cage reached 50% of

the standardized density that we used for that volume of cage. Flies were briefly anesthetized using carbon dioxide during these consolidations. At the beginning of this mortality assay, the CO and nCO populations had been under C-type selection for 341 and 88 generations, respectively. The SCO-a and SCO-b populations had completed 58 generations of intense selection.

3.3 Statistical Methods

Analysis for Physiological Characters

Each of the five measured phenotypes, glycogen content, lipid content, water content, starvation resistance, and desiccation resistance, were analyzed separately. We now outline the two linear mixed-effects models we used for starvation resistance.

The first model was used for our within selection regime comparisons. Let y_{ijk} be the measured starvation resistance for selection treatment-i (i=1 (CO or Sco-a) and 2 (nCO or Sco-b)), population-j (j= 1..10), and individual-k (k=1..n_l). Then the effects of the fixed and random effects can be modeled as,

$$y_{ijk} = \mu + \alpha\delta_i + \beta_j + \varepsilon_{ijk} \quad (1)$$

where δ_i=0 if i=1, and 1 otherwise.

The second model was used for our selection type comparisons (i.e. C-type selection vs. S-type selection). Let y_{ijk} be the measured starvation resistance for selection type-i (i=1 (C type) and 2 (S type)), population-j (j= 1..20), and individual-k (k=1..n_l). Then the effects of the fixed and random effects can be modeled as,

$$y_{ijk} = \mu + \alpha\delta_i + \beta_j + \varepsilon_{ijk} \quad (2)$$

where δ_i=0 if i=1, and 1 otherwise.

For these two models, the main effects of selection regime and replicate population are measured by α and β, respectively. The different populations contribute random effects to these measurements by genetically based differences that arise due to random genetic drift and are measured by β while individual random variation is measured by ε. Both sources of random variation are assumed to be independent normally distributed random variables with zero means. The model parameters were estimated with the R *lme* function (R Core Team, 2015).

Cochran-Mantel-Haenszel (CMH) tests were used to analyze the rates of cardiac arrests between two different stocks (i.e. CO_i vs nCO_i). The CMH test is used when there are repeated tests of independence, or multiple 2x2 tables of independence. This is the equation for the CMH test statistic, with the continuity correction included, that we used for our statistical analyses:

$$X^2_{\mathrm{MH}} = \frac{\{|\Sigma\left[a_i - \frac{(a_i + b_i)(a_i + c_i)}{n_i}\right]| - 0.5\}^2}{\Sigma(a_i + b_i)(a_i + c_i)(b_i + d_i)(c_i + d_i)/(n_i^3 - n_i^2)}$$

We designated "a" and "b" as the number of cardiac arrests in population *i* of the first stock and population *i* of the second stock, respectively. We designated "c" and "d" as the number of contracting hearts in the two populations. The n_i represents the sum of a_i, b_i, c_i, and d_i. The subscript *i* (i = 1..5), representing one of the five replicate populations within each of the six stocks.

Analysis for Age-Specific Mortality Rates

We first tested for convergence within the selection treatments (i.e. CO vs. nCO, and SCO-a vs. SCO-b) for effects of selection on mortality rates over the adult lifespan. The

observations consisted of mortality rates at a particular age (t) but within a small age interval (*k*-1, 2,…, m). These age intervals were chosen to span the ages, such that all comparison populations still had live flies. Within each interval, mortality rates were modeled by a straight line and allowing selection regime (*i*=1 (CO or SCO-a), *i*=2 (nCO or SCO-b)) to affect the intercept of that line but not the slope. Slopes were allowed to vary between intervals. Populations (*j*=1,…,10) were assumed to contribute random variation to these measures. Let y_{ijkt} be the measured mortality rate at age-*t*, interval-*k*, selection treatment-*i*, population-*j*. Then the effects of the fixed and random effects can be modeled as,

$$y_{ijkt} = \alpha + \beta_k + \delta_i\gamma_i + (\omega + \pi_k\delta_k)t + \delta_k\delta_i\mu_{ik} + c_j + \varepsilon_{ijkt} \quad (3)$$

where δ_i=0 if *i*=1, and 1 otherwise.

We then tested for divergence between the selection treatments (i.e. C-type selection vs. S-type selection). Let y_{ijkt} be the measured mortality rate at age-t, interval-k, selection treatment type-*i* (*i*=1 (C type) and 2 (S type)), population-*j* (*j*= 1..20). Then the effects of the fixed and random effects can be modeled as

$$y_{ijkt} = \alpha + \beta_k + \delta_i\gamma_i + (\omega + \pi_k\delta_k)t + \delta_k\delta_i\mu_{ik} + c_j + \varepsilon_{ijkt} \quad (4)$$

where δ_i=0 if *i*=1, and 1 otherwise.

In equations 3 and 4, the c_j and ε_{ijkt} are independent standard normal random variables. The different populations contribute random effects to these measurements by genetically based differences that arise due to random genetic drift and are measured by *c* while individual random variation is measured by ε. Both sources of random variation are assumed to be independent normally distributed random variables with zero means. The model parameters were estimated with the R *lme* function (R Core Team, 2015).

IV. Results

4.1 Starvation Resistance and Lipid Content

The S-type populations had a significantly higher average starvation survival time and average lipid content than the C-type populations (see Fig. 3.1 and Fig. 3.2). First off, the average survival time during starvation, or starvation resistance, for the five SCO-a populations is 150.98 hours, and for the five SCO-b populations it is 152.2 hours. The starvation resistance of the SCO-a and SCO-b populations are statistically similar (p-value = 0.9501). The average lipid content for the five SCO-a populations is 0.138 mg per fly, while the average lipid content for the five SCO-b populations at 14 days of age from egg is 0.143 mg per fly. The lipid content between these two sets of S-type populations were also statistically similar (p-value = 0.754). The five CO populations have a much lower average starvation survival time of 66.84 hours, and an average lipid content of 0.058 mg per fly at 14 days of age. The recently-derived nCO populations had an average starvation survival time of 61.4 hours, and an average lipid content of 0.051 mg per fly. The CO and nCO populations are not significantly different in regards to starvation resistance (p-value = 0.081) and lipid content (p-value = 0.367). After finding no significant differences within the two selection types, we compared all ten C-type populations against the ten S-type populations. The S-type populations have a significantly higher average lipid content than the C-type populations by 0.086 mg/fly (p-value < 0.0001). Additionally, the S-type populations have a significantly greater starvation survival time than the C-type populations, with a difference of 87.44 hours (p-value = 0.01).

In an unpublished experiment from our lab, we had taken samples of CO, nCO, SCO-a and SCO-b female flies to measure lipid content during the age-specific mortality assay at three separate ages (day 14, 21, and 28 from egg; see Fig. 3.7). The C-type populations had an

average lipid content of 0.049 mg/fly at age 14, while the S-type populations had an average lipid content of 0.125 mg/fly at age 14 (p-value < 0.0001). The C-type populations increased from 0.049 mg/fly to 0.1 mg/fly in one week (p-value < 0.0001), and maintain a similar lipid content at age 28. The S-type populations also significantly increased from 0.125 mg/fly at age 14 to 0.36 mg/fly by age 21 (p-value < 0.0001), and continued to 0.43 mg/fly by age 28 (p-value < 0.0001). These unpublished results and our age-specific mortality rates presented in this study provided the basis for measuring age-specific lipid content and age-specific cardiac arrest rates at ages 14 through 49 days from egg.

When looking at age-specific lipid contents, both the CO and SCO-a sets of populations increased in lipid content from age 14 to age 21 (see Fig. 3.8). The five CO populations went from an average 0.068 mg/fly lipid content to 0.103 mg/fly in one week (p-value = 0.019). However, the CO populations maintained a stable average lipid content (~ 0.10 mg/fly) from age 21 through age 49. At age 14 from egg, the SCO-a populations had an average lipid content of 0.129 mg/fly, which significantly increased to 0.397 mg/fly by age 21 from egg (p-value < 0.0001). The average lipid content for the SCO-a populations continued to rise through age 35, reaching an average lipid content of 0.584 mg/fly (p-values < 0.0001). At all ages, the S-type populations had a significantly higher average lipid content than the C-type populations (p-value < 0.01).

4.2 Desiccation Resistance, Water Content, and Glycogen Content

Similarly to starvation resistance and lipid content, we found significantly lower average water content and average glycogen contents in the C-type populations when compared to S-type populations (see Figs. 3.4 and 3.5). The CO and nCO populations have similar average water

contents of 0.919 ml/fly and 0.863 ml/fly (p-value = 0.114). The five SCO-a and five SCO-b populations had average water contents of 1.058 ml/fly and 1.094 ml/fly, respectively (p-value = 0.374). When we compared the 10 S-type populations with the 10 C-type populations, the average water content of the S-type populations was slightly significantly higher than the average water content of the C-type populations (p-value = 0.031). The average glycogen contents for the CO and nCO populations were 0.0684 mg/fly and 0.0762 mg/fly, respectively (p-value = 0.366). The average glycogen contents for the SCO-a and SCO-b populations were 0.151 mg/fly and 0.163 mg/fly, respectively (p-value = 0.473). When we compared the 10 C-type populations with the 10 S-type populations, we found that the S-type populations had a larger average glycogen content than the C-type populations by 0.0844 mg/fly (p-value < 0.001).

With water content and glycogen content being major factors in desiccation resistance, it was not surprising to find parallel results for desiccation resistance among these populations (see Fig. 3.3). The five CO and five nCO populations have average desiccation survival times of 14.71 hours and 14.63 hours, respectively. These two sets of C-type populations were not significantly different from each other (p-value: 0.935). The five SCO-a populations and the five SCO-b populations had average desiccation survival times of 17.29 hours and 16.34 hours, respectively. These two sets of S-type populations were not significantly different from each other (p-value = 0.278). When we compared the 10 S-type populations against the 10 C-type populations, the S-type populations had a higher average desiccation survival time by 2.14 hours. However, our linear mixed effects analysis showed this difference was not statistically significant (p-value = 0.0697).

4.3 Cardiac Arrest Rates

Our first set of experiments compared the C and S-type populations at age 14 days from egg. The S-type populations clearly have a dysfunctional cardiac system in comparison to the C-type populations (See Fig. 3.6). The five CO populations had an average arrest rate of 25.3%, whereas the five nCO populations had an average arrest rate of 27.3% (p-value = 0.643). The five SCO-a populations had an average arrest rate of 54%, whereas the five SCO-b populations had an average rate of 52.7% (p-value = 0.806). With no significant differences found within the two selection treatments, we then compared the cardiac arrest rates of the 10 S-type populations against the 10 C-type populations. The 10 C-type populations had an average cardiac arrest rate of 26.33%, whereas the 10 S-type populations had an average cardiac arrest rate of 53.33%. The average cardiac arrest rate of the S-type populations was significantly higher than the C-type populations (p-value = 2.37×10^{-21}).

In our second set of experiments, at ages 14, 21, and 28 days from egg, the five SCO-a populations had an average rate of cardiac arrests significantly higher than that of the five CO populations (p-value < 0.05; see Fig. 3.9). It was not until age 35 from egg where the cardiac arrest rates in the SCO-a populations (57.69%) were no longer significantly different than the CO populations (59.4%; p-value = 0.826). The lack of a significant difference in arrest rates was also observed at age 42 and age 49 from egg.

4.4 Age-Specific Mortality Rates

The age-specific mortality rates of the C-type populations and the S-type populations significantly differ starting at age 20 days from egg, continuing through age 40 days from egg (see Fig. 3.10). The mortality rates of the S-type populations dramatically diverge from the pre-

aging plateau shown by the C-type populations. This rapid increase in S-type mortality rates is seen from age 14 to 25 days from egg, and is then followed by a roughly stable mortality through age 40 days from egg. The mortality rates of the C-type populations converge on the rates of the S-type populations during the 41-43 day age-interval (p-value = 0.129). The mortality rates of the 10 S-type populations mirror those of the 10 C-type populations for the remainder of the assay. After the "aging phase" ends, the twenty populations show the "late-life" plateau that has been documented before in our outbred *Drosophila* populations (e.g. Rose et al. 2002; Burke et al. 2016).

V. Discussion

5.1 Extreme Differentiation Between C-type and S-type Populations

With only 67 generations of intense selection for increased starvation resistance, the S-type populations have clearly differentiated from their ancestors in many of the physiological assays mentioned above. As seen in other work in our laboratory and our colleagues' laboratories, it was not surprising to find such extreme differentiation in starvation resistance and lipid content between the C-type and S-type populations (vid. Hardy et al. 2015; Rose et al. 1992). The 10 S-type populations survived starvation an average of 87.52 hours longer than the 10 C-type populations. Additionally, the average S-type fly had a larger lipid content than a C-type fly by 0.859 mg. It has been well documented that starvation resistance and lipid content are two characters that are highly correlated with each other (Djawdan et al. 1998; Hardy et al. 2015). Once again, we found a strong, positive correlation between lipid content and starvation resistance among these 20 populations ($R^2 = 0.8598$).

Implementing an intense selection protocol also indirectly affected multiple other physiological characters. Selecting for increased starvation resistance has also increased the water content, glycogen content, and desiccation resistance in the S-type populations. The average water content between the S-type and C-type populations were significantly different. We had expected there to be an increase in water content, however, we did not expect the difference to be statistically significant after only 67 generations of selection. In Archer et al. (2007), they intensely selected for increased desiccation resistance for 37 generations in *Drosophila melanogaster* populations (NDO) and compared the water content, water loss rates, glycogen content, and desiccation resistance of those populations to another set of desiccation resistant populations (D), which had been under intense selection for 184 generations. They found that in just 37 generations of intense selection, the NDO populations had similar levels of water loss rates and glycogen content as the D populations. However, water content and overall desiccation resistance were still significantly different, showing that desiccation resistance is a more complex trait than starvation resistance. In 1988, our lab observed a slow return of desiccation resistance to ancestral levels in a reverse selection experiment (Service et al. 1988). In Passananti et al. (2004), they also found that desiccation resistance decreases at a much slower rate during relaxed selection of the Rose Lab's intensely selected desiccation resistant lines. With that said, it was surprising to find a significant difference in water content, and almost a significant difference in desiccation resistance, despite those two traits only being indirectly selected on for under 70 generations. We found a strong, positive correlation between glycogen content and desiccation resistance within these 20 populations ($R^2 = 0.7023$), as well as between water content and desiccation resistance ($R^2 = 0.6315$).

5.2 Drosophila as a Model for Heart and Obesity-Related Disorders

Human obesity has grown to epidemic proportions in the United States, with excessive lipid accumulation being a risk factor for metabolic disorders and heart disease. High-fat diets (HFDs) and excessive lipid accumulation are believed to be the major contributors to this problem. With cardiovascular disease and heart-related defects being leading causes of death among Western patients, conducting useful heart experiments at great scale and intensity with model organisms should be of significant value (Olson 2004).

Experimentally probing *D. melanogaster* physiology via selection experiments has improved our understanding of this complex metazoan as an experimental model for the study of health and disease. Unlike most microbial models, the physiology of *Drosophila* is both complex and broadly analogous to some features of vertebrate physiology. *D. melanogaster* also have homologous genetic mechanisms with those that are thought to determine lifespan among vertebrates, including such genetic systems as TOR and insulin/insulin-like signaling (e.g. Partridge and Gems 2007). With that said, *Drosophila* cardiac function and its relationship to other physiological processes has been of great scientific interest recently.

There are important differences between *Drosophila* and mammalian cardiovascular structure and function. *Drosophila* have an open circulatory system and a reversible direction of hemolymph flow. There is also no relationship between the circulatory system and the respiratory system in *Drosophila*. However, there are similarities in (a) early heart development, (b) age-dependent decline in heart function, and (c) the genes associated with heart development, function, and diseases (Bier and Bodmer 2004; Bodmer and Venkatesh 1998; Cripps and Olson 2002; Diop and Bodmer 2012; Nishimura et al. 2011; Ocorr et al. 2007; Zaffran and Frasch 2002). In 2004, Bier and Bodmer examined age-dependent decline in cardiac function in

Drosophila by showing that adult responses to external electrical pacing decline with age. Additionally, *Drosophila* shares some of the genes that underlie its cardiac performance with those of human cardiac genetics, such as *tinman* (Bodmer 1993; Bodmer 2006) and *opa1* (Shahrestani et al. 2009). The fruit fly has become a powerful tool for understanding cardiomyopathies, metabolic homeostasis, and other obesity-related disorders (Diop and Bodmer 2015; Smith et al. 2014; Trinh and Boulianne 2013).

Starvation-resistant and obese *Drosophila* populations are a powerful tool not only for studying the evolution of starvation responses, but also for studying metabolic disorders and related cardiac dysfunction (Birse et al. 2010; Diop and Bodmer 2012; Hardy et al. 2015; Smith et al. 2014; Trinh and Boulianne 2013). Increased lipid content can be achieved by (1) selection for starvation resistance or (2) exposure to a high-fat diet. In a similar experimental setup as ours, Hardy et al. (2015) observed dilated hearts and reduced contractility in their evolved obese populations after 65 generations of selection for starvation resistance. Fruit flies with increased lipid content display hyperglycemia, insulin resistance, reduced cardiac contractility, and cardiac arrhythmias (Birse et al. 2010; Hoffmann et al. 2013; Na et al. 2013; Trinh and Boulianne 2013). These comparable characteristics to those of mammalian metabolic syndrome further support the value of using *Drosophila* for heart studies.

Our results suggest that the increased lipid levels due to increased starvation resistance weaken cardiac function, specifically the ability to recover from a fibrillation-like event. The ten S-type populations had an average cardiac arrest rate of 53.33% under electrical pacing, whereas the ten C-type populations only had an average rate of 26.333% (Figure 3.6). The stark contrast in rate of cardiac arrests is not hard to comprehend, especially when you consider the 0.09 mg difference of lipid content and 86 hour increase in starvation resistance between the S-type and

C-type populations at age 14 days. This difference then worsens with age. We found a strong, positive correlation between lipid content and cardiac arrest rates within these 20 populations (R^2 = 0.7885). Additionally, if you take into account both lipid and glycogen content, we found an even stronger correlation between the total energy content and cardiac arrest rates (R^2 = 0.882). The S-type populations appear to display similar cardiac dysfunction as the experimental populations in the studies previously referenced.

The salience of these cardiological differences for overall survival is underscored by the substantially increased mortality rates shown by the starvation-selected populations during early adulthood. While this difference eventually goes away at later adult ages, it is also often found that the effect of obesity on human mortality rates shows a similar age dependence. Specifically, the optimal BMI levels for human mortality appear to increase with adult age (Flegel et al. 2013; Ford et al. 2014; Jackson et al. 2014). Thus, even though there is a complex age-dependent relationship between lipid levels and mortality rates in obese fruit flies, it echoes some features of the relationship between obesity and mortality in human populations.

Through intense selection for starvation resistance, we have created a set of ten large, outbred, obese *Drosophila* populations that have a dramatic increase in lipid content and other metabolic reserves. These visibly obese populations exhibit a decline in heart robustness in relation to its ancestral CO populations, a decline that is associated with increased mortality rates. The use of multi-omic tools with these populations could help parse the genetic and molecular underpinnings of heart performance, disease, and other obesity-related disorders. Only a few cases of human heart disease arise from single-locus mutations (i.e. *opa1*; Shahrestani et al. 2009). Other types of heart disease may involve many genes and thus many biochemical pathways. The types of heart disease that are prevalent among present-day human populations

are unlikely to result from deleterious alleles of major effect. It is therefore useful to study heart function in large outbred populations of *D. melanogaster* that differ in allele frequencies at many loci. The SCO populations described here could be an invaluable resource in understanding the most common forms of heart disease and other obesity-related disorders.

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VIII. Figures and Figure Legends

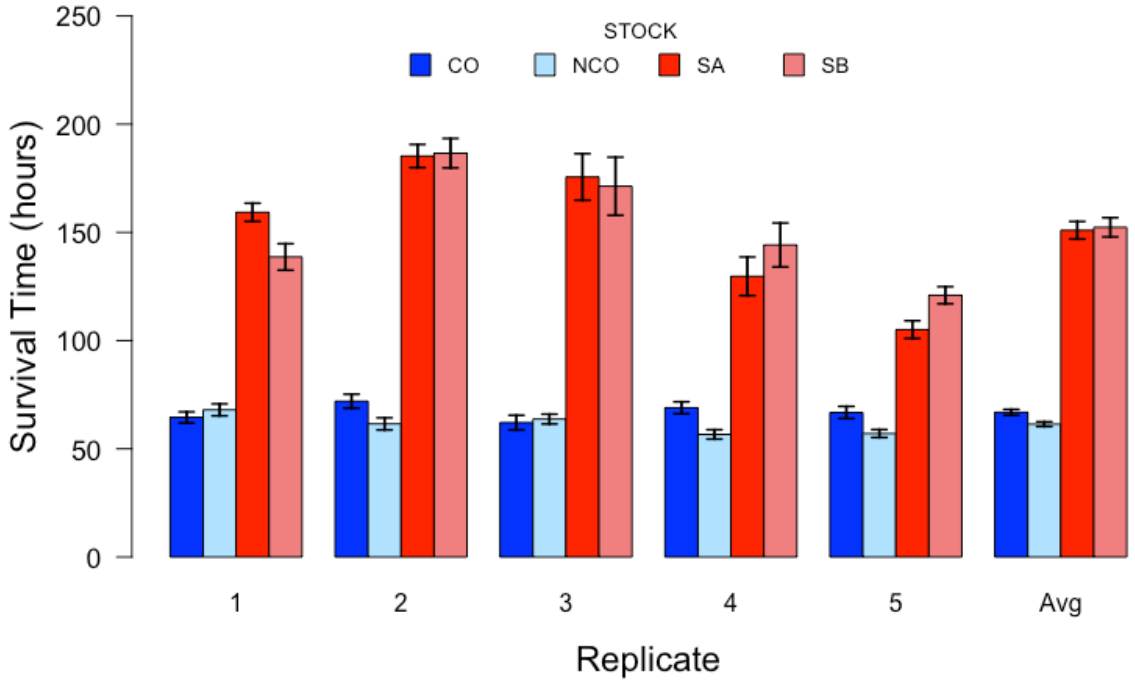

Figure 3.1. The average survival time of female fruit flies at 14 days from egg in a starvation environment for the four five-fold replicated stocks (mean ± 1 SEM). SCO-A (SA) and SCO-B (SB) populations had significantly longer survival times than the CO and nCO populations (p-value = 0.01).

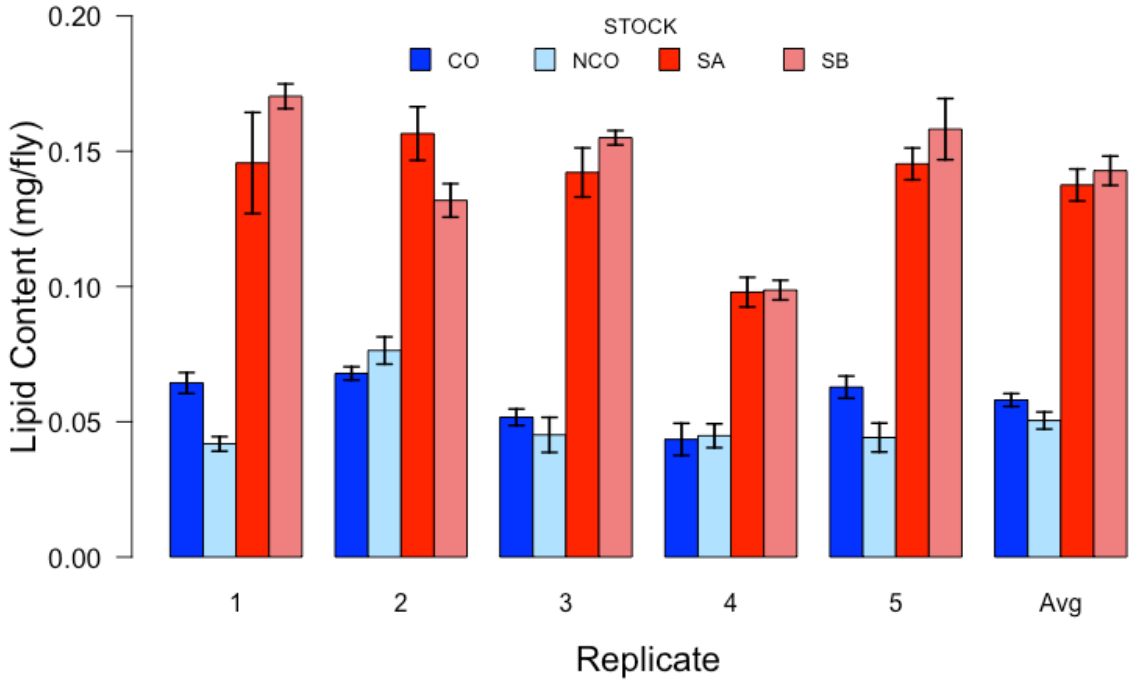

Figure 3.2. The average lipid content of female fruit flies at 14 days from egg from the four five-fold replicated stocks (mean ± 1 SEM). SCO-a (SA) and SCO-b (SB) populations had significantly larger average lipid contents than the CO and nCO populations (p-value < 0.0001).

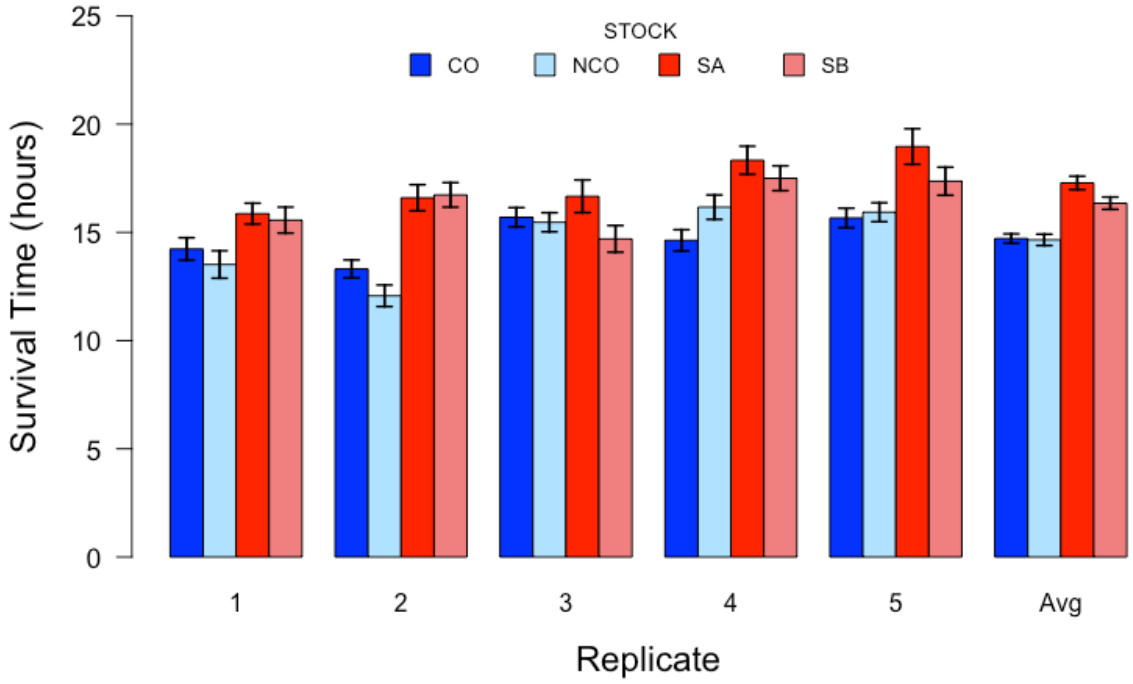

Figure 3.3. The average survival time of female fruit flies at 14 days from egg in a desiccation environment for the four five-fold replicated stocks (mean ± 1 SEM). Despite the average survival times of the SCO-a (SA) and SCO-b (SB) populations being slightly longer than the average survival time of the CO and nCO populations, the difference was barely insignificant (p-value = 0.0697).

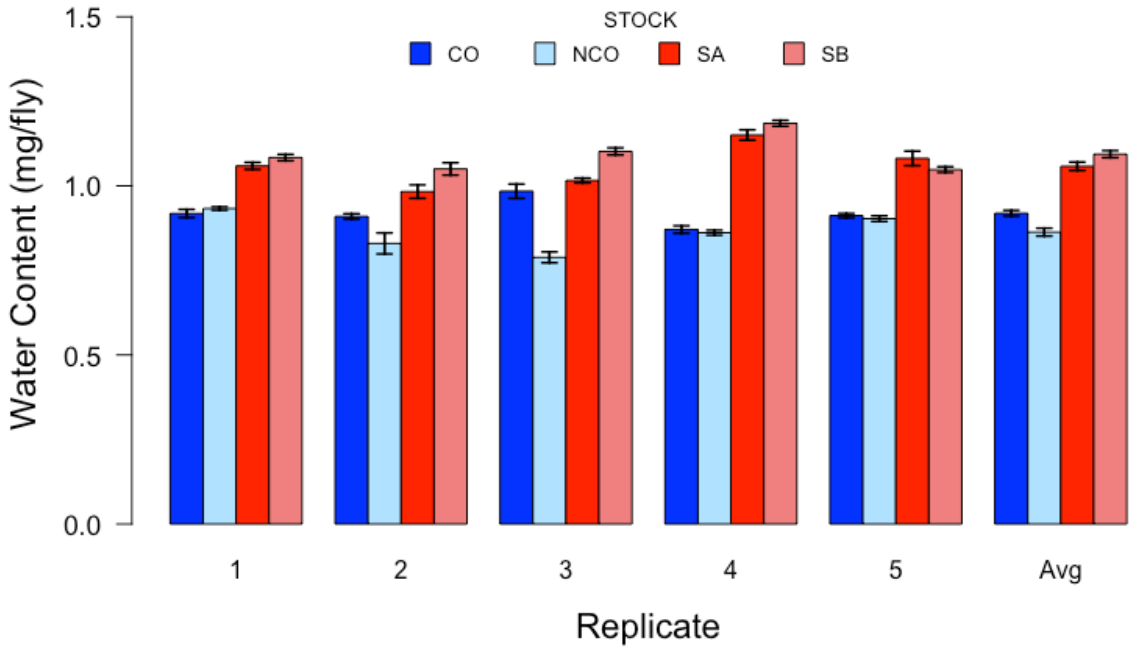

Figure 3.4. The average water content of female fruit flies at 14 days from egg from the four five-fold replicated stocks (mean ± 1 SEM). SCO-a (SA) and SCO-b (SB) populations had significantly larger average water contents than the CO and nCO populations (p-value = 0.031).

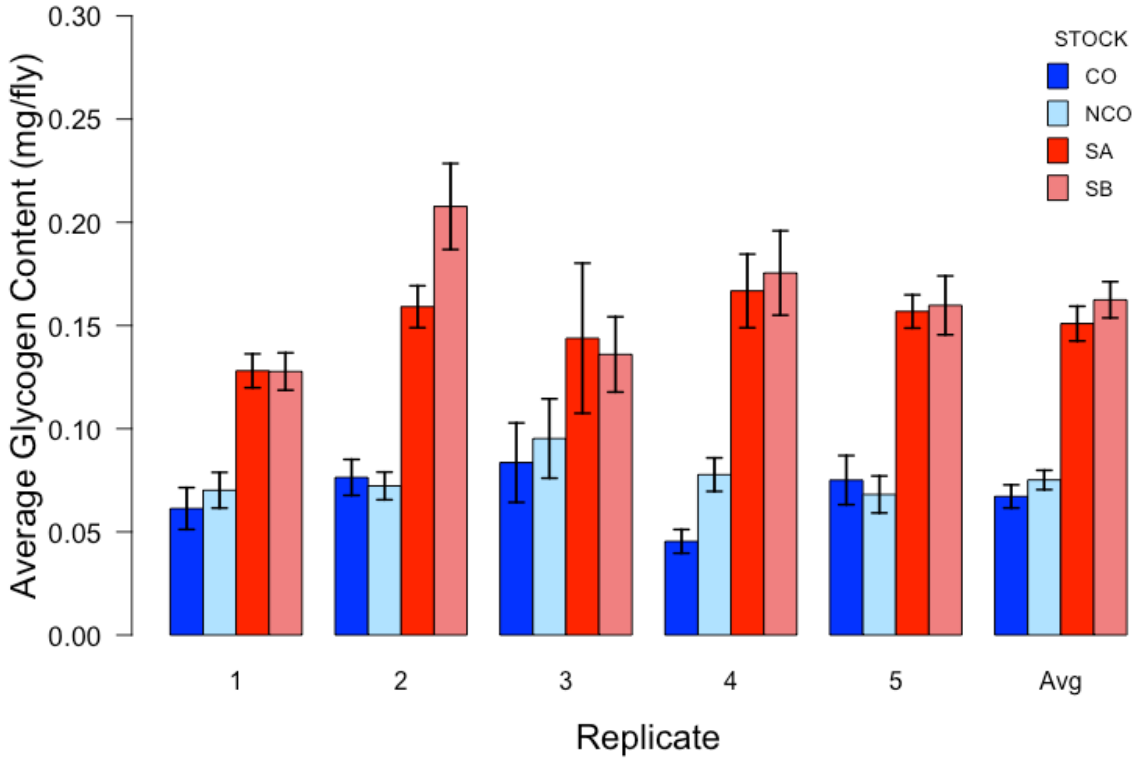

Figure 3.5. The average glycogen content of female fruit flies at 14 days from egg from the four five-fold replicated stocks (mean ± 1 SEM). SCO-a (SA) and SCO-b (SB) populations had significantly larger average glycogen contents than the CO and nCO populations (p-value < 0.001).

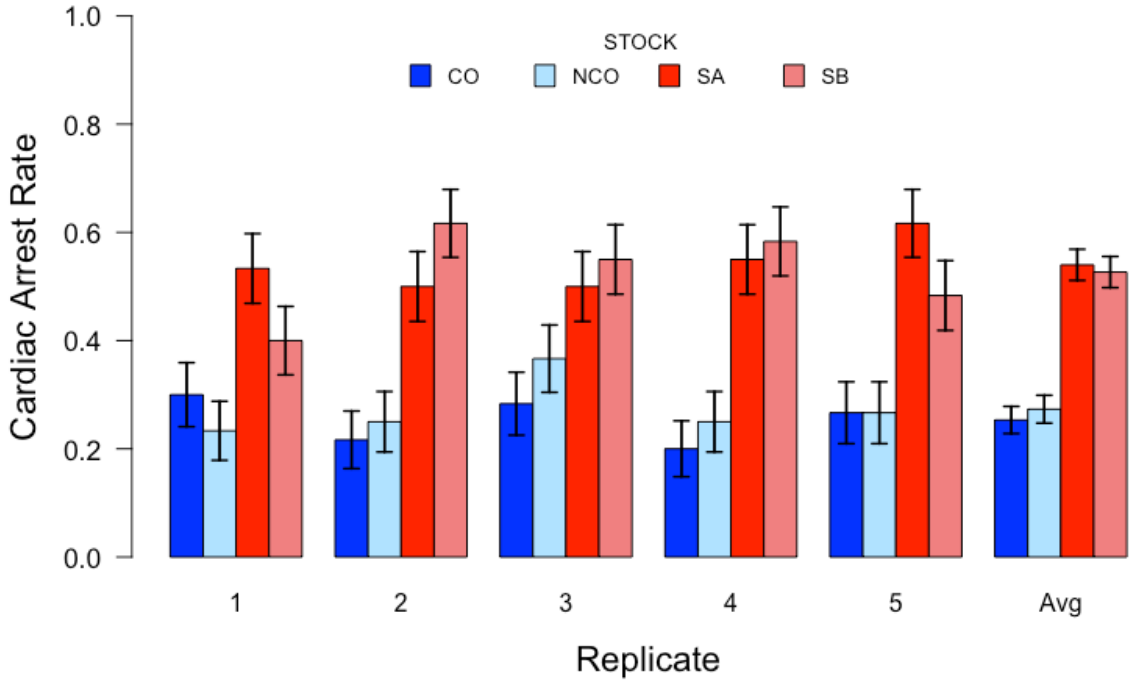

Figure 3.6. The average rate of cardiac arrests of female fruit flies at 14 days from egg from the four five-fold replicated stocks (mean ± 1 SEM). The SCO-a (SA) and SCO-b (SB) populations had higher average rates of cardiac arrests than the CO and nCO populations (p-value = 2.37×10^{-21}).

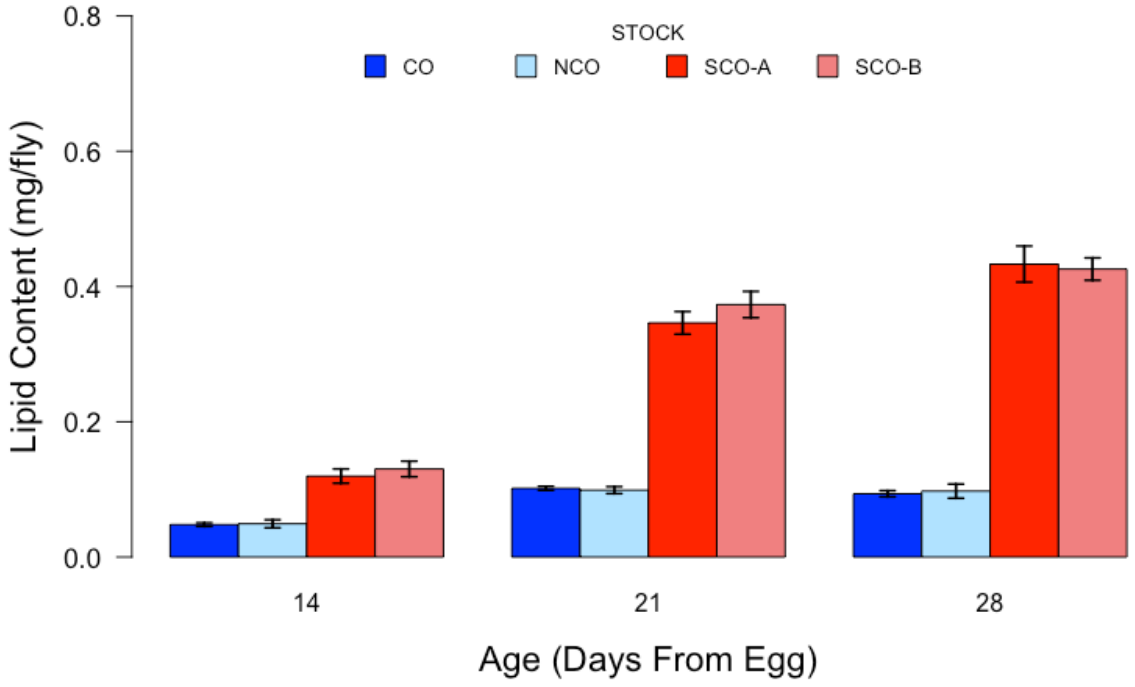

Figure 3.7. The average lipid contents of the ten C-type and ten S-type populations at ages 14, 21, and 28 days from egg. The C-type populations significantly increased from 0.049 mg/fly to 0.1 mg/fly in one week (p-value < 0.0001), and maintain a similar lipid content at age 28. The S-type populations significantly increased from 0.125 mg/fly at age 14 to 0.36 mg/fly by age 21 (p-value < 0.0001), and continued to 0.43 mg/fly by age 28 (p-value < 0.0001).

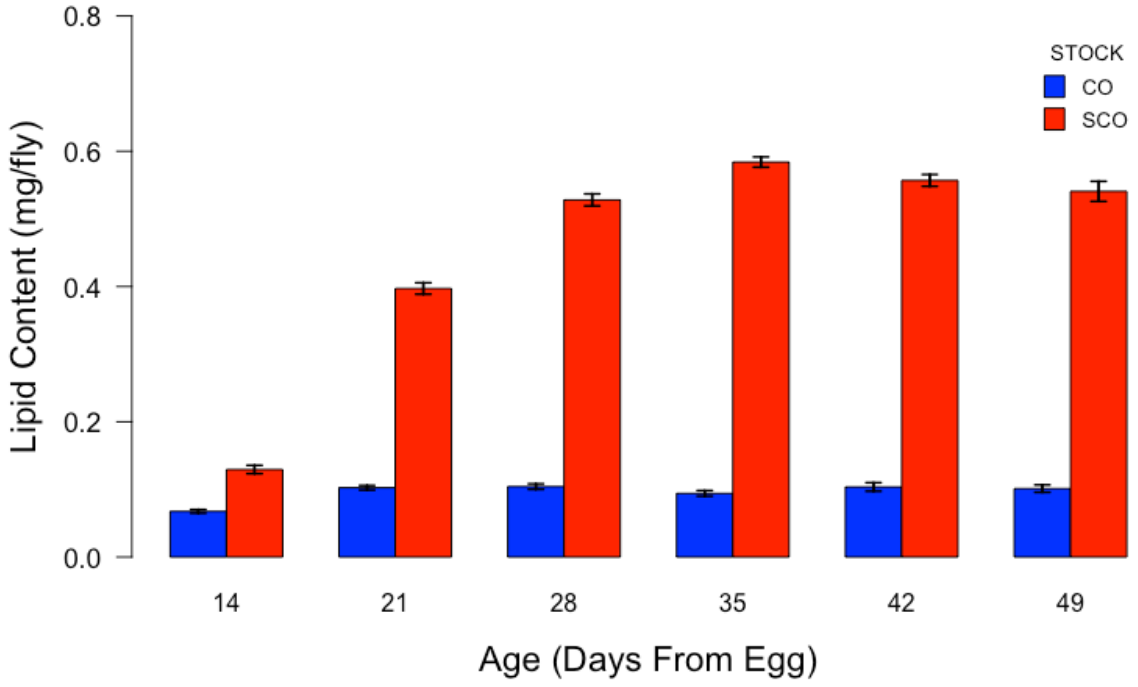

Figure 3.8. The age-specific average lipid contents of female fruit flies from the five CO populations and five SCO-a populations at six different ages (mean ± 1 SEM). The SCO populations had statistically significantly, larger average lipid contents than the CO populations at all six ages.

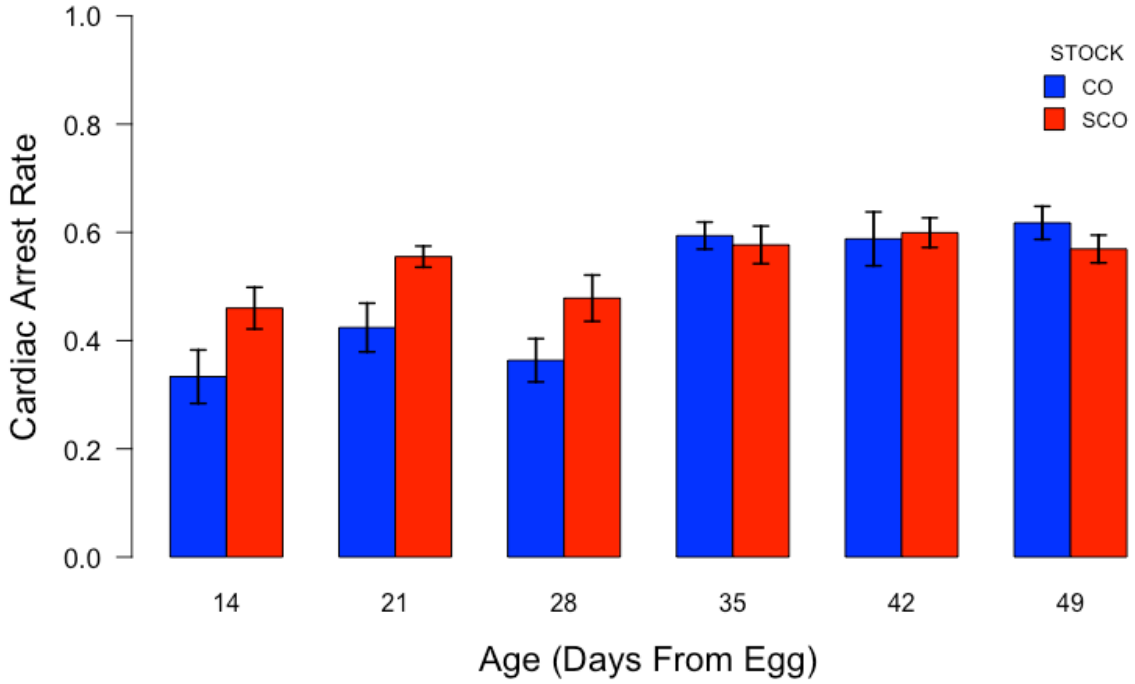

Figure 3.9. The age-specific average rate of cardiac arrests of female fruit flies from the five CO and five SCO-a populations at six different ages (mean ± 1 SEM). The SCO populations had significantly higher average rates of cardiac arrests than the CO populations at ages 14, 21, and 28 days from egg. At ages 35, 42, and 49 days from egg, the average rate of cardiac arrests are no longer statistically significantly different.

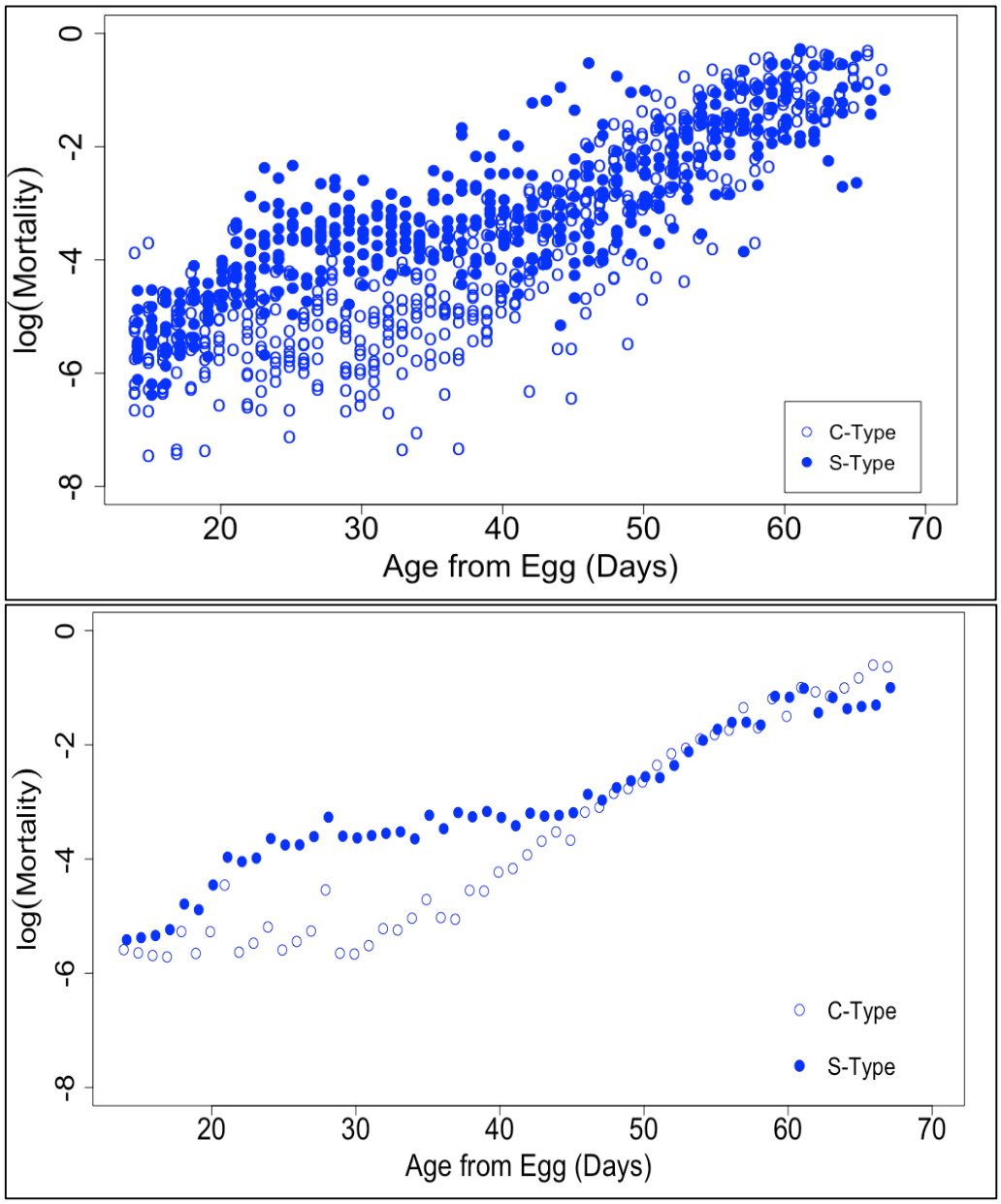

Figure 3.10. (Top) The age-specific mortality rates for each of ten C-type and ten S-type populations. **(Bottom)** The average age-specific mortality rate of the C-type populations and the S-type populations. The S-type populations have a significantly higher age-specific mortality rate than the C-type populations from ages 20 to 40 days from egg (p-values < 0.05). The mortality rates of the C-type populations converge on the rates of the S-type populations during the 41-43 day age-interval (p-value = 0.129).

CHAPTER 4

The Effects of High-Fat, High-Caffeinated Diets on *Drosophila melanogaster*

I. Abstract

Cardiovascular disease and heart defects are one of the leading causes of death in modern-day human populations. The spread of high-fat diets (HFDs) among human populations around the world has been epidemiologically associated with the spread of metabolic disorders such as type two diabetes and heart disease. This in turn provides strong motivation for research on heart disease in model organisms such as *Drosophila*, which are currently the simplest model organism that can be used to examine heart function. Previous studies in our lab have shown that (1) fruit flies briefly subjected to starvation sometimes have a *reduced* rate of cardiac arrest when electrically paced, and (2) fruit flies selected for starvation resistance, and thus much fatter, have an *increased* rate of cardiac arrest when electrically paced. In effect, this experiment seeks to emulate the effects of chronic consumption of the high-fat, high-caffeine fast-food diet by incorporating coconut oil and caffeine into the fly food medium. Both of our short-lived and long-lived fruit fly populations that were exposed to a high-fat diet, had an increased rate of cardiac arrest. Exposure to aspirin and caffeine produced insignificant effects on cardiac arrest rates and mortality rates. Through various manipulations of lipid storage and intake, our lab has continued to build on the strong, adverse relationship between lipid content and heart robustness in *Drosophila melanogaster*. Our study provides additional support for the use of *Drosophila* in experiments focused on revealing physiological and genetic machinery of vertebrates and invertebrates affected by the consumption of a high-fat diet.

II. Introduction

With cardiovascular disease and heart-related defects being leading causes of death among Western patients, conducting useful heart experiments at great scale and intensity with model organisms should be of significant value (Olson 2004). Human obesity has grown to epidemic proportions in the United States, with excessive lipid accumulation being a risk factor for metabolic disorders and heart disease. Not surprisingly, the spread of such high fat diets (HFDs) among human populations around the world has been associated with the increasing rate of obesity.

Drosophila is now a common invertebrate system of choice to study heart development, function, and aging, as well as obesity-related disorders. The tube-like heart structure in *Drosophila* separates this model species from other common invertebrate model species, making *Drosophila* the simplest model organism that can be used for heart function studies (Bier and Bodmer 2004). Furthermore, *Drosophila* have short generation times, are easily maintained at large population sizes, and possess vast public genomic resources (e.g. Burke and Rose 2009). This makes them a powerful invertebrate model system with which to study heart development, heart function, and heart aging (Bier and Bodmer 2004; Nishimura et al. 2011; Ocorr et al. 2007; Trinh and Boulianne 2013)

Fruit flies experience a decline in heart function and robustness with age, similar to what has been found in aging human adults (Bier and Bodmer 2004; Nishimura et al. 2011; Ocorr et al. 2007; Paternostro et al. 2001; Wessells and Bodmer 2007). This is just one of many parallels between heart disease in *Drosophila* and humans. It has also been shown that cardiac arrhythmia increases with adult age in *Drosophila* (Ocorr et al. 2007; Wessells and Bodmer 2004), just as it does in humans. Additionally, there is an important adverse effect of body fat on heart function

in fruit flies, a parallel result to those of many comparable studies in human cardiology (Crewe et al. 2013; Heinrichsen and Haddad 2012; Manrique et al. 2013).\

But the research connections between fruit fly hearts and human hearts are deeper than functional parallels (Diop and Bodmer 2012; Smith et al 2014; Trinh and Boulianne 2013). The *D. melanogaster* heart has been used as a model for identifying genes that cause heart disease (e.g. Wolf et al. 2006). The genetics of heart formation and disease in vertebrates are conserved in *Drosophila* genetics. For example, *Drosophila* heart formation is dependent on the gene *tinman* (Bodmer 1993; Bodmer et al. 1990). Without the expression of *tinman*, not only does the heart not form, but other visceral and dorsal skeletal muscles do not form (Bodmer and Venkatesh 1998). In vertebrate species, two genes related to *tinman* are *Nkx2-3* and *Nkx2-5*. However, the dysfunction created via knockout mutations of these vertebrate genes occurs later in the developmental stages of the heart. The vertebrate heart tube fails to loop properly due to the lack of (1) bHLH protein eHAND (Biben and Harvey 1997) and (2) the Ankyrin-repeat protein CARP (Zou et al. 1997). Vertebrate *Nkx2-5* mutants do not suffer a complete absence of heart formation, unlike *Drosophila tinman* mutants. Other vertebrate genes and transcription factors associated with heart function and development that are conserved are: *MEF2 genes*, *BMP* genes, bHLH proteins, and GATA transcription factors (reviewed in Bodmer and Venkatesh 1998).

Two approaches can be taken to study cardiovascular disease and other obesity-related disorders in *Drosophila*: (1) creating a set of obese populations via selection (e.g. Hardy et al. 2015), or (2) exposing populations to high-fat or high-sugar diets (e.g. Birse et al. 2010). Hardy et al. (2015) found that after 65 generations of selection for starvation resistant, their evolved obese populations displayed cardiac dysfunction. Their starvation resistant *Drosophila* have (i)

dilated hearts and (ii) reduced contractility. This documented cardiac dysfunction has been attributed to larger lipid droplets impinging on the anatomical position of the heart (Hardy et al. 2015). Similarly to Birse et al. (2010), Hardy found that a prolonged period of fasting rescued *Drosophila* heart function in their experiments. These comparable characteristics to those of mammalian metabolic syndrome further support the value of using *Drosophila* for heart studies.

The effects of a HFD on cardiac function in *Drosophila* were extensively detailed by Birse et al. (2010). Fruit flies fed a HFD had (i) increased overall triglyceride fat content, (ii) increased cardiac lipid accumulation, (iii) reduced cardiac contractility, (iv) blocked heart electrical conduction, and (v) severe structural pathologies. An intriguing finding of theirs is that either overexpression of lipase or the reduction of insulin-TOR activity lower lipid accumulation during HFD exposure. They then show that such reduced lipid accumulation in turn mitigates the adverse effects of HFD on cardiac fat accumulation and cardiac function. Still other genes and signaling pathways conserved between *Drosophila* and mammals can counteract the lipotoxicity and cardiac dysfunction of obesity (e.g. *PGC-1/spargel*: Diop et al. 2015).

Given the mounting evidence for an adverse effect of lipid accumulation on heart health, we too were interested in observing the effects of a HFD on our short-lived and long-lived outbred *Drosophila* populations. These populations are qualitatively different from the inbred and mutant flies used in many laboratories, with higher levels of heterozygosity, fecundity, stress resistance, and other measures of health and fitness. That is, we believe our *Drosophila* populations are genetically analogous to human populations in a way that inbred and mutant lines are not. But we are also interested in combining HFDs with other dietary stressors, which might impinge on heart function. One such substance that we have studied in our lab and in collaboration with other labs is caffeine (e.g. Matsagas et al. 2009). Caffeine shows a dose-

dependent toxic effect on survival, fecundity, and other functions in *Drosophila* (Matsagas et al. 2009; Nikitin et al. 2008; Potdar et al. 2017). Likewise, very high levels of caffeine consumption are associated with health problems in human populations, including heart disease (Cornelis and El-Sohemy 2007). We were also interested in seeing how aspirin affects our populations, especially those receiving the high-fat diet. Aspirin is an anti-inflammatory chemical that also reduces the formation of blood clots. This chemical could potentially help relieve the likely adverse effects of a high fat diet on *Drosophila* heart performance. The experiment we report here tried to emulate the effects of chronic consumption of the high-fat, high-caffeine fast-food diet using otherwise robust outbred *Drosophila* populations. Here we looked at the potential effects of high fat, high caffeinated diets on the heart robustness, longevity, and fecundity of ten experimentally-evolved populations that were never systematically inbred or mutagenized.

III. Materials and Methods

3.1 Experimental material

This study employed 10 of the large, outbred, and highly differentiated populations created by the Rose laboratory over more than 30 years (Rose 1984; Rose et al. 1992; Rose et al. 2004). These populations have been selected for reproduction at different ages in the life cycle: 10 days (ACO_{1-5} populations) and 28 days (CO_{1-5} populations). [See Chippindale et al. (1997) for details concerning the creation of ACO stocks; Rose et al. 1992 for the creation of the CO stocks; Rose (1984)]. All of the populations assayed here descend from a single *Drosophila melanogaster* population, called IV. The B_{1-5} populations (baseline) and O_{1-5} (10-week generation cycle) were derived from the single IV population in 1980. In 1989, the CO_{1-5}

populations were derived from the O_{1-5} populations. In 1991, the ACO_{1-5} populations, which "A" stands for accelerated, were derived from the CO_{1-5} populations.

3.2 Rearing protocols

Two run-in generations, the first being 12 days and the second being 14 days, were used to remove any parental or grand-parental epigenetic effects. The populations were cultured in regular banana-molasses food from egg to adult, on a 24L:0D light schedule. Populations were maintained and assayed in a laboratory kept at 23 – 25 °Celsius. Eggs were collected at a density of 60 to 80 eggs per vial after adults were allowed 24 hours to lay eggs. At the end of each run-in generation (day 12 and day 14), the populations were transferred to an acrylic cage. Replicate populations of the same number (e.g. ACO_1 and CO_1) were handled in parallel at all stages. On day 14 of the second run-in generation, the adults were assigned at random to one of the dietary treatments.

3.3 Dietary Treatments

Standard Yeast Paste (Y)

For populations receiving the control diet, a 100 x 15 mm petri dish filled with agar was placed in the cages with a smaller petri dish containing our standard yeast paste. The standard yeast paste is made of 25 grams of active dry yeast, 40 milliliters of deionized water, and 2 milliliters of 1% acetic acid.

High-Fat Diet (HFD)

For populations receiving the HFD, a 100 x 15 mm petri dish filled with agar was placed in the cages with a smaller petri dish containing our standard yeast paste mixed with coconut oil. Ten milliliters of virgin coconut oil replaced 10 milliliters of deionized water.

Caffeine Diet (CA)

For populations exposed to caffeine, a 100 x 15 mm petri dish filled with agar was placed in the cages with a smaller petri dish containing our standard yeast paste mixed with caffeine. A caffeine dose of 10x the daily standard fruit fly dose was mixed with 1.5 milliliters of deionized water. The 1.5 milliliters caffeine-water solution was added to 38.5 milliliters of deionized water before being mixed in with 25 grams of active dry yeast and two milliliters of 1% acetic acid.

Aspirin Diet (ASP)

For populations exposed to aspirin, a 100 x 15 mm petri dish filled with agar was placed in the cages with a smaller petri dish containing our standard yeast paste mixed with aspirin. An aspirin dose of 10x the daily standard fruit fly dose was mixed with 1.5 milliliters of deionized water. The 1.5 milliliters aspirin-water solution was added to 38.5 milliliters of deionized water before being mixed in with 25 grams of active dry yeast and two milliliters of 1% acetic acid.

High-Fat Diet with Caffeine (HFD-CA)

For populations exposed to the high-fat diet supplemented with caffeinated, a 100 x 15 mm petri dish filled with agar was placed in the cages with a smaller petri dish containing our standard yeast paste mixed with coconut oil and caffeine. A caffeine dose of 10x the daily

standard fruit fly dose was mixed with 1.5 milliliters of deionized water. The 1.5 milliliters caffeine-water solution was added to 28.5 milliliters of deionized water. Ten milliliters of coconut oil was then added to the caffeine-water solution before being mixed in with 25 grams of active dry yeast and two milliliters of 1% acetic acid.

High-Fat Diet with Aspirin (HFD-ASP)

For populations exposed to the high-fat diet supplemented with aspirin, a 100 x 15 mm petri dish filled with agar was placed in the cages with a smaller petri dish containing our standard yeast paste mixed with coconut oil and aspirin. An aspirin dose of 10x the daily standard fruit fly dose was mixed with 1.5 milliliters of deionized water. The 1.5 milliliters aspirin-water solution was added to 28.5 milliliters of deionized water. Ten milliliters of coconut oil was then added to the caffeine-water solution before being mixed in with 25 grams of active dry yeast and two milliliters of 1% acetic acid.

3.4 Experimental Assays

Electrical Pacing Assay

On day 14 from egg, 40 female flies from each replicate population were chosen at random to undergo cardiac pacing. Female flies from each replicate per stock were first chosen at random. The flies were anesthetized for three minutes using triethylamine, also known as FlyNap, and then placed on a microscope slide prepared with foil and two electrodes. FlyNap was chosen as the anesthetic because of its minimal effect on heart function and heart physiology when administered for more than one minute (Chen and Hillyer 2013). The cold-shock method was not used as an anesthetic for the cardiac pacing assay, because the flies need to be fully

anesthetized throughout the procedure. If the flies regain consciousness, the added stress and abdominal contractions while trying to escape would alter heart rate and function more than FlyNap does. Paternostro et al. (2001) found that FlyNap has the least cardiac disruption compared to the two other substances commonly used for *Drosophila* anesthesia, carbon dioxide and ether. Two electrodes were attached to a square-wave stimulator in order to produce electric pacing of heart contraction. Anesthetized flies were attached to the slide between the foil gaps using a conductive electrode jelly touching the two ends of the fly body, specifically the head and the posterior abdomen tip. The shocking settings for this assay were 40 volts, six Hertz, and 10 ms pulse duration. Each shock lasted for 30 seconds. An initial check of the status of the heart was made after completion of the shock, followed by a check after a two-minute "recovery" period. Heart status was scored as either contracting or in cardiac arrest. The protocol for this assay is outlined in Wessells and Bodmer (2004).

Adult Mortality and Fecundity

Flies from the ACO and CO populations were handled in parallel for two generations in vials. After 14 days of development in vials, approximately 500 adult flies from each of the ACO and CO populations were transferred into Plexiglass cages. Each population had 12 cages containing 500 flies each. Two cages of 500 flies from each population were assigned one of the dietary treatments mentioned above. Dead flies were collected from cages and counted at the same time every day. Mortality data were obtained over adult ages from day 14 to day 28 from egg.

Fecundity was measured in concert with the mortality assay. After removing dead flies from each cage, a new agar plate with the designated dietary treatment was placed in the cage.

The eggs that had been laid on the surface of the agar plate removed after mortality were collected through a filtration process. After the eggs were collected on a black fabric membrane, a digital image of the membrane was then taken, and the number of eggs laid were counted using computer software ImageJ.

3.5 Statistical Analysis

Cardiac Arrest Rates

Cochran-Mantel-Haenszel (CMH) tests were used to analyze the rates of cardiac arrests between two different dietary treatments within a stock system (i.e. HFD-ACO$_i$ vs Y-ACO$_i$). The CMH test is used when there are repeated tests of independence, or multiple 2x2 tables of independence. This is the equation for the CMH test statistic, with the continuity correction included, that we used for our statistical analyses:

$$X^2_{\text{MH}} = \frac{\{|\Sigma\left[a_i - \frac{(a_i + b_i)(a_i + c_i)}{n_i}\right]| - 0.5\}^2}{\Sigma(a_i + b_i)(a_i + c_i)(b_i + d_i)(c_i + d_i)/(n_i^3 - n_i^2)}$$

We designated "a" and "b" as the number of cardiac arrests in population *i* of the first stock and population *i* of the second stock, respectively. We designated "c" and "d" as the number of contracting hearts in the two populations. The n_i represents the sum of a_i, b_i, c_i, and d_i. The subscript *i* (i = 1..5), representing one of the five replicate populations within each of the six stocks.

Adult Mortality and Fecundity

The age-specific survival probability *(p_x)* is the probability of a female surviving to an age *x* given that she survived to the previous age and is calculated by the following equation:

$$p_x = 1 - \left(\frac{d_x}{n_x}\right)$$

where d_x is the number of females that die at age *x,* and n_x is the number of females that were alive at the start of age *x.* Age-specific fecundity (m_x) is the average number of eggs laid per surviving female at age *x*. The product of these two variables gives an estimate of how cohorts are functioning at each age. In our experiments, the unit interval for *x* is a single day.

We tested for differences in adult mortality rates and $p_x m_x$ over 14 consecutive ages. The observations consisted of either the mortality rate, or $p_x m_x$ at an age (*x*). This was modeled by a straight line allowing diet (*j*=1,…,6) to affect the intercept, but not the slope of the line. Populations (*i* = 1, 2…,6) and duplicated cohort cages (*k* = 1 or 2), contributed random variation to these measures. With the notation above, the $p_x m_x$ at age (*x*), diet (*j*), population (*i*), cohort (*k*) is y_{ijkx} and can be described by,

$$y_{ijkx} = \alpha + \delta_j \beta_j + \omega x + \gamma_i + \psi_{(i)k} + \varepsilon_{ijkx},$$

where $\delta_s = 0$ if *s* = 1, and 1 otherwise, and γ_i, $\psi_{(i)k}$, and $\mathcal{E}_{ijkx}$ are independent standard normal random variables with variances σ_γ^2, σ_ψ^2, and σ_ε^2, respectively. The effects of diet on the intercept are assessed by considering the magnitude and variance of γ_i, $\psi_{(i)k}$, and β_j. The different populations contribute random effects to these measurements by genetically based differences that arise due to random genetic drift and are measured by γ and ψ while individual random variation is measured by ε. Both sources of random variation are assumed to be independent normally distributed random variables with zero means. The model parameters were estimated with the R *lme* function (R Core Team, 2015).

IV. Results

4.1 Effects on Cardiac Arrest Rates

Exposure to a HFD caused significant cardiac dysfunction across all ten of our short-lived and long-lived outbred *Drosophila* populations (see Fig. 4.1 and Fig. 4.2). On average, the five ACO experimental populations that received the HFD had a 42% higher rate of cardiac arrest when compared to the control populations (p-value = 0.009). The average rate of cardiac arrest for ACO populations exposed to the HFD was 47.2%. The average rate of cardiac arrest for the ACO populations receiving the control diet was 33.2%. For the CO experimental populations, the average rate of cardiac arrest was double in comparison to the CO control populations (34.5% versus 17%, respectively). This increase of cardiac arrests in the HFD exposed CO populations was statistically significantly (p-value < 0.001).

Unlike the exposure to coconut oil, exposure to caffeine or aspirin did not have a significant impact on the rates of cardiac arrest across the ten ACO and CO populations (see Figs. 4.3-4.6). On average, the five ACO populations that received the caffeinated yeast paste had a rate of cardiac arrest 4% lower than the rate seen in the control populations (p-value = 0.877; see Fig. 4.3). The average rate of cardiac arrest for ACO populations exposed to the caffeine was 31.9%. The average rate of cardiac arrest for the ACO populations receiving the control diet was 33.2%. For the CO caffeinated populations, the average rate of cardiac arrest was nearly identical in comparison to the CO control populations (19.33% versus 17%, respectively; see Fig. 4.4). This slight increase of cardiac arrests in the caffeinated CO populations was not statistically significant (p-value = 0.596).

The ACO populations that received the yeast paste with aspirin had a cardiac arrest rate 6.1% higher than the cardiac arrest rate of the control populations (p-value = 0.721). The

average rate of cardiac arrest for ACO populations exposed to the aspirin was 35.3% (see Fig. 4.5). The average rate of cardiac arrests for the ACO populations receiving the control diet was 33.2%. For the CO populations receiving the aspirin diet, the average rate of cardiac arrest was 23.7% (see Fig. 4.6). However, the increased rate of cardiac arrests due to aspirin diet was not significantly higher than the rate seen in the control CO populations (17%; p-value = 0.152).

The high-fat diet treatment with caffeine supplementation significantly increased the rate of cardiac arrests in the ACO populations (see Fig. 4.7). The average rate of cardiac arrest for the HFD-CA exposed ACO populations was 46.2%. Whereas, the average rate of cardiac arrest for the control ACO populations was 33.2%. This dietary treatment also significantly increased the rate of cardiac arrests in the ACO populations (p-value = 0.013). For the CO experimental populations, the average rate of cardiac arrest was almost double in comparison to the CO control populations (33.6% versus 17%, respectively). This increase in rate of cardiac arrests in the HFD-CA exposed CO populations was statistically significant (p-value < 0.001; see Fig. 4.8).

The high-fat diet treatment with aspirin supplementation significantly increased the rate of cardiac arrests in the ACO populations (see Fig. 4.9). The average rate of cardiac arrest for the HFD-CA exposed ACO populations was 44.8%. Whereas, the average rate of cardiac arrest for the control ACO populations was 33.2%. This increase in rate of cardiac arrests was statistically significant (p-value = 0.035). For the CO experimental populations, the average rate of cardiac arrest was more than double in comparison to the rate of cardiac arrests in the CO control populations (36.4% versus 17%, respectively). This dietary treatment also significantly increased the rate of cardiac arrests in the ACO populations (p-value < 0.001; see Fig. 4.10).

4.2 Effect on Mortality

First, we measured the effects of these dietary treatments on mortality rates within each set of populations over a 14-day period. The effects of these six different dietary treatments on survivorship are similar between the two sets of populations. We observed an adverse impact on survivorship in the ACO populations receiving a dietary treatment that contained coconut oil (i.e. HFD, HFD-CA, and HFD-ASP; see Fig. 4.11). A significant increase in mortality rates is also seen in the five CO populations that received a the HFD and the HFD-Asp treatment (see Fig. 4.12). The HFD-CA dietary treatment did not produce a significantly higher mortality rate compared to the control, caffeine, or aspirin dietary treatments in the CO populations. We found no differences in mortality rates between the control diet (Y), the aspirin diet (ASP), and the caffeine diet (CA) in both ACO and CO populations.

The second linear mixed effects model was used to measure the effect on one dietary treatment on survivorship in our ten ACO and CO populations, against the effect of another dietary treatment on survivorship in these same ten populations over a 14-day period. Once again, we see significant increases in mortality rates (p-values < 0.0001) when the ten populations are given a dietary treatment containing the coconut oil component (i.e. HFD, HFD-ASP, and HFD-CA). In comparison to the mortality rates of ten populations that received the control yeast diet, populations receiving the aspirin and caffeine diets did not have a significantly different mortality rate (p-values > 0.9).

4.3 Effect on Fecundity

First, we measured the effects of these dietary treatments on fecundity within each of the $ACO_{1\text{-}5}$ and the $CO_{1\text{-}5}$ populations. The effects of these six different dietary treatments on

fecundity are slightly different between the two sets of populations (see Fig. 4.13 and Fig. 4.14). The ACO populations receiving the aspirin dietary treatment had the highest $p_x m_x$ values (e.g. ACO Y vs. ACO ASP: p-value = 0.008). The aspirin dietary treatment produced significantly higher $p_x m_x$ values than the other five treatments. The caffeine treatment led to the lowest $p_x m_x$ values in the ACO populations. This $p_x m_x$ values of ACO populations receiving caffeine were only significantly lower than the $p_x m_x$ values of populations receiving the HFD and HFD-ASP treatments (p-values < 0.05). Of the five experimental dietary treatments, only the aspirin dietary treatment significantly affected the $p_x m_x$ value in relation to the control (Y) dietary treatment.

The $p_x m_x$ values are also the lowest in the CO populations that received the caffeine dietary treatment (see Fig. 4.14). This dietary treatment significantly lowered the $p_x m_x$ values in comparison to the control treatment (p-value = 0.0001). Unlike the ACO populations, the aspirin dietary treatment does not produce the highest $p_x m_x$ values in the CO populations. However, populations that received the HFD-ASP diet had the highest $p_x m_x$ values. This HFD-ASP treatment was the only diet to produce significantly higher $p_x m_x$ values than the control (Y) diet (p-value = 0.034). Only the caffeine and HFD-ASP dietary treatments significantly affected the $p_x m_x$ values in relation to the control (Y) dietary treatment.

The second linear mixed effects model was used to measure the effect of one dietary treatment on $p_x m_x$ in our ten ACO and CO populations, against the effect of another dietary treatment on $p_x m_x$ in these same ten populations over an 11-day period (17-28 days from egg). The caffeine dietary treatment produced the lowest $p_x m_x$ values among the 10 populations. This diet was the only treatment that had significantly lower $p_x m_x$ values compared to the control diet

(p-value < 0.001). The HFD-ASP dietary treatment was the only dietary treatment that significantly increased $p_x m_x$ values compared to the control diet (p-value = 0.044).

V. Discussion

Exposure to a diet containing coconut oil leads to increased rates of cardiac arrest in our $ACO_{1\text{-}5}$ and $CO_{1\text{-}5}$ populations. In our long-lived CO populations, the HFD, HFD-ASP, and HFD-CA dietary treatments all produced significantly higher rate of cardiac arrest (p-value < 0.05). We also found the same significant effects in our ACO populations (p-value < 0.05). The higher rates of cardiac arrest can be attributed to the potential elevated lipid content accumulated during the four-day exposure. Our results suggest that increased lipid levels could cause cardiac dysfunction, specifically the ability to recover from a fibrillation-like event. We hypothesize that the elevated lipids are accumulating in the hemolymph, creating a more viscous solution that is more difficult to pump or producing lipid blockages in the *Drosophila* heart, perhaps both.

Needless to say, the dietary treatments containing the coconut oil component also increased the mortality rates in the ACO and CO populations. The control, aspirin, and caffeine diets all produced the lowest mortality rates in our populations. Exposing our CO populations to the HFD and HFD-ASP also led to higher $p_x m_x$ values, although not significantly higher than the control yeast diet. It is possible that these two high fat diets forced a reallocation of nutrients and energy from somatic cell repair to egg production. Either the shift of energy from the fat bodies to the ovaries, or the increased energy from breaking down the increased lipid intake could explain the increased $p_x m_x$ values. It is worth noting that exposure to the caffeine diet produced significantly lower $p_x m_x$ values in our $CO_{1\text{-}5}$ populations, despite lowering the rates of mortality.

Caffeine also lowered the $p_x m_x$ values in our $ACO_{1\text{-}5}$ populations, but it was not significantly lower compared to populations receiving the controlled yeast diet.

Our results corroborate the findings for high-fat diet studies from the Burnham Institute (Birse et al. 2010; Diop and Bodmer 2012; Diop et al. 2015). Additionally, these results align neatly with two other experiments in our lab. The first experiment looked at how brief periods of starvation affected heart robustness in *Drosophila* (Kezos et al. 2017). We found a decreased rate of cardiac arrest after a brief period of starvation. The second experiment intensely selected for increased starvation resistance in derivatives of our CO populations. After 90 generations of intense selection, those starvation-selected populations have an increased starvation resistance of 100 hours, increased lipid content of 0.07 mg/fly at age 14 from egg, and doubled rate of cardiac arrest compared to their ancestor populations. From all of these results, it is apparent that a diet that features an increased level of fat is detrimental for physiological functions. That still leaves some mechanistic questions unanswered, especially the role of hemolymph viscosity.

A further step for research on heart function in *Drosophila* would be to examine the impact of adaptation to a high-fat diet over the course of experimental evolution. We have done a variety of experiments of this kind before, in which we have selected for fly adaptation to (i) mild starvation, (ii) intense starvation, (iii) intense desiccation. We are hopeful that selection on heart robustness under high-fat dietary conditions will likewise reveal additional physiological and genetic machinery required to tolerate modern high-fat diets, given the many promising parallels and homologies between heart disease in humans and fruit flies. Such research would not, of course, fully resolve the mechanistic foundations of human heart disease, given the genetic and physiological differences between fruit flies and humans. But, as in other chronic disease conditions, fruit flies would provide an additional view of the molecular, genetic and

other machinery underlying heart disease. Sequencing such populations genome-wide could identify the genomic foundations for developing resistance to the adverse effects of such diets.

VI. Acknowledgements

We thank James W. Hicks and Timothy J. Bradley for helpful discussions and comments on the experiment. We thank Laurence D. Mueller and Thomas T. Barter for their assistance with the statistical analysis and interpretation. We are grateful to the many undergraduate research students who contributed to the stock maintenance and experimental assays, especially Kenneth J. Arnold, Jenny Lu, Danh Vu, Isaias Sanchez, Chloe Nouzille, Kristen Hanna, Jacqueline Ghaly, Bishoy Hanna, and Kylina Trinh.

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VIII. Figures and Figure Legends

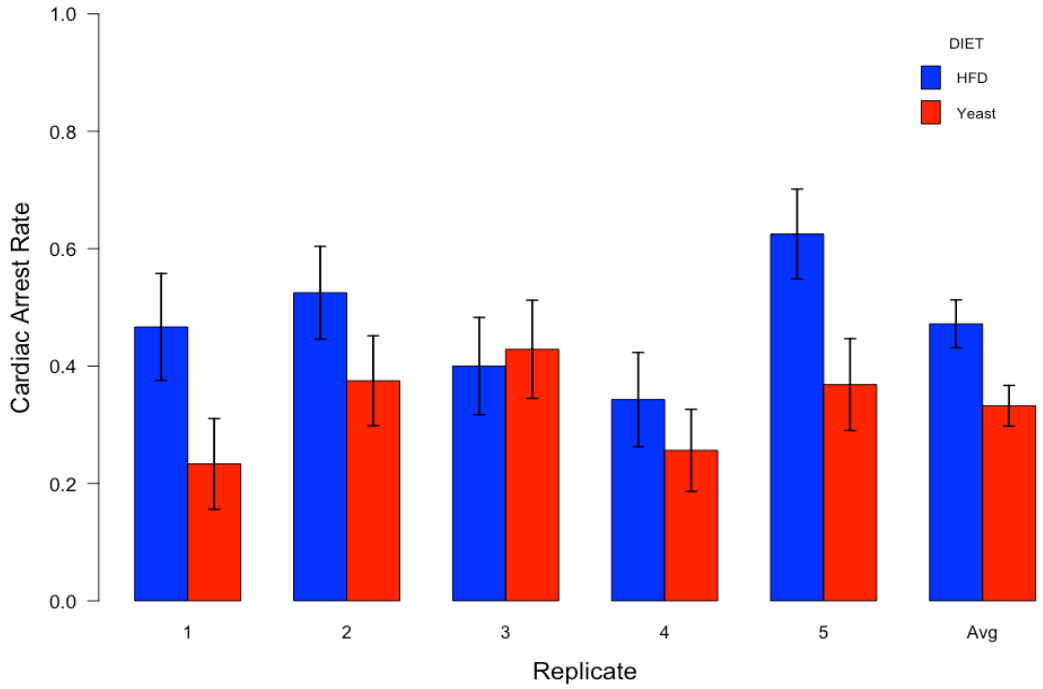

Figure 4.1. The average rate of cardiac arrests of female fruit flies from our $ACO_{1\text{-}5}$ populations (mean ± 1 SEM). The populations fed a high-fat diet (HFD) had higher average rate of cardiac arrests than the ACO populations receiving the controlled yeast diet (p-value = 0.0093).

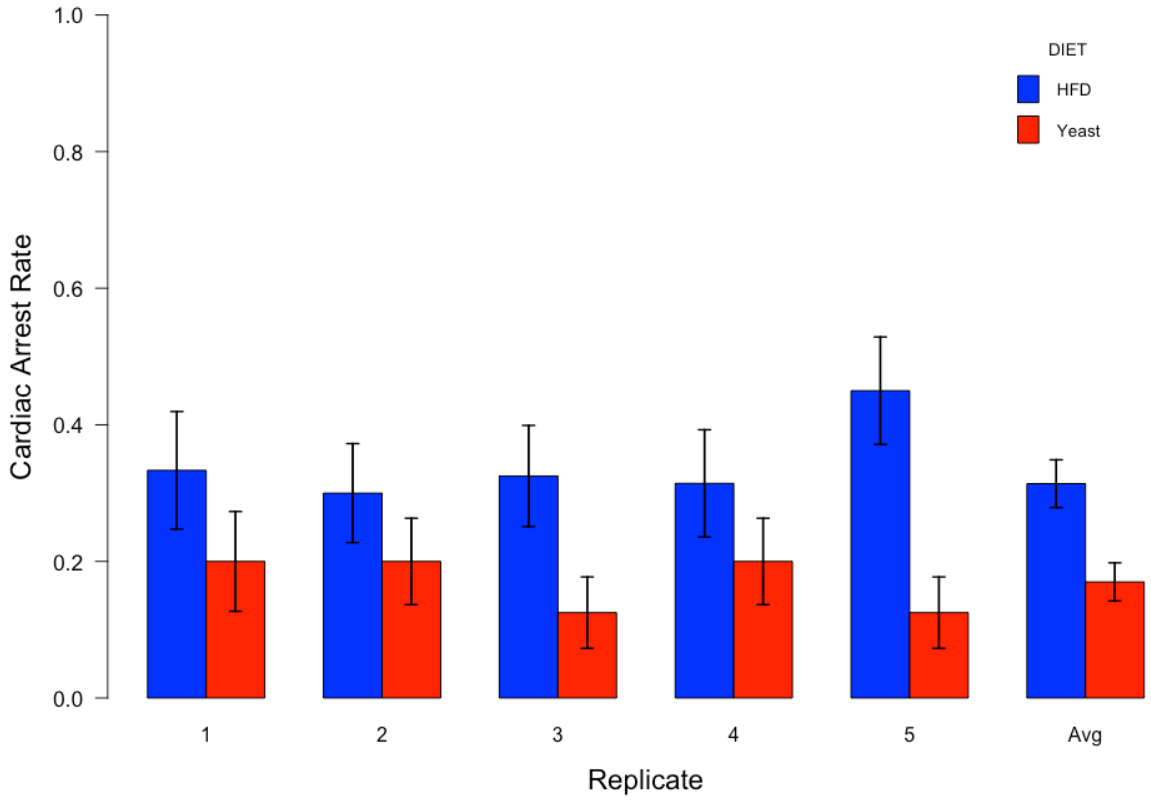

Figure 4.2. The average rate of cardiac arrests of female fruit flies from our $CO_{1\text{-}5}$ populations (mean ± 1 SEM). The populations fed a high-fat diet (HFD) had higher average rate of cardiac arrests than the CO populations receiving the controlled yeast diet (p-value < 0.001).

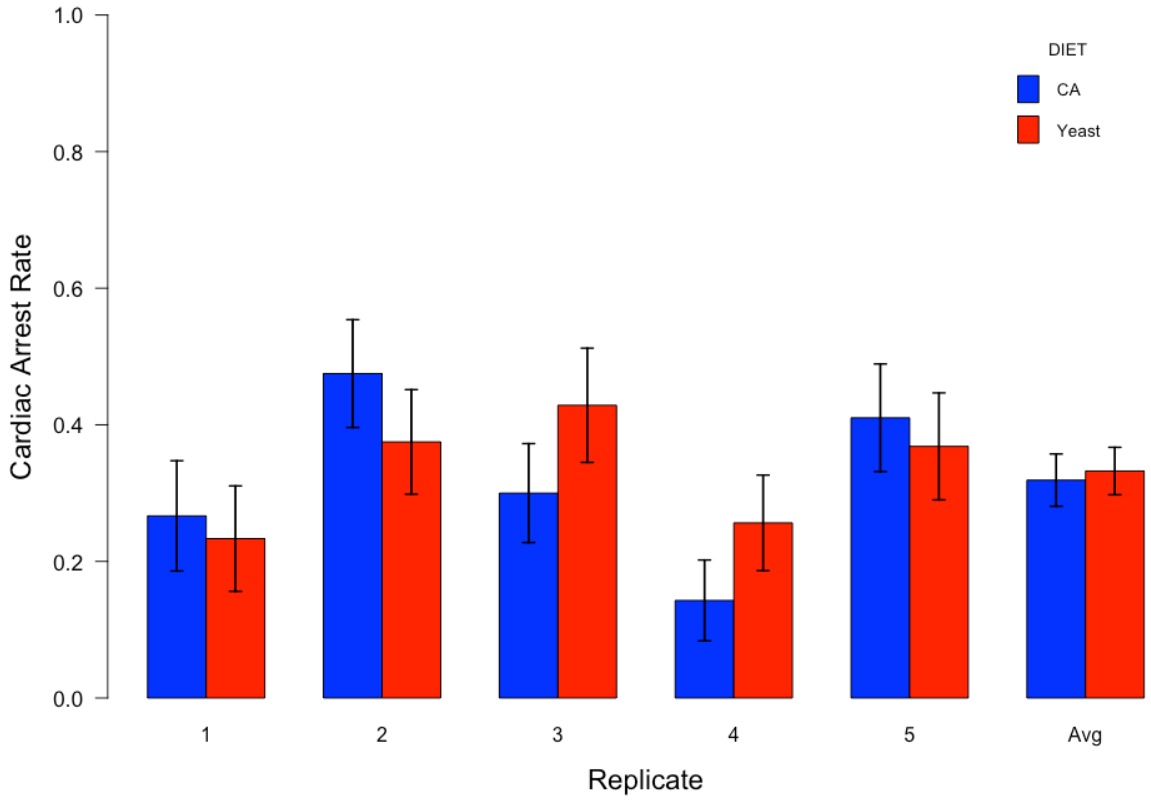

Figure 4.3. The average rate of cardiac arrests of female fruit flies from our $ACO_{1\text{-}5}$ populations (mean ± 1 SEM). The populations fed a caffeinated diet (CA) did not have significantly different average rate of cardiac arrests than the ACO populations receiving the controlled yeast diet (p-value = 0.877).

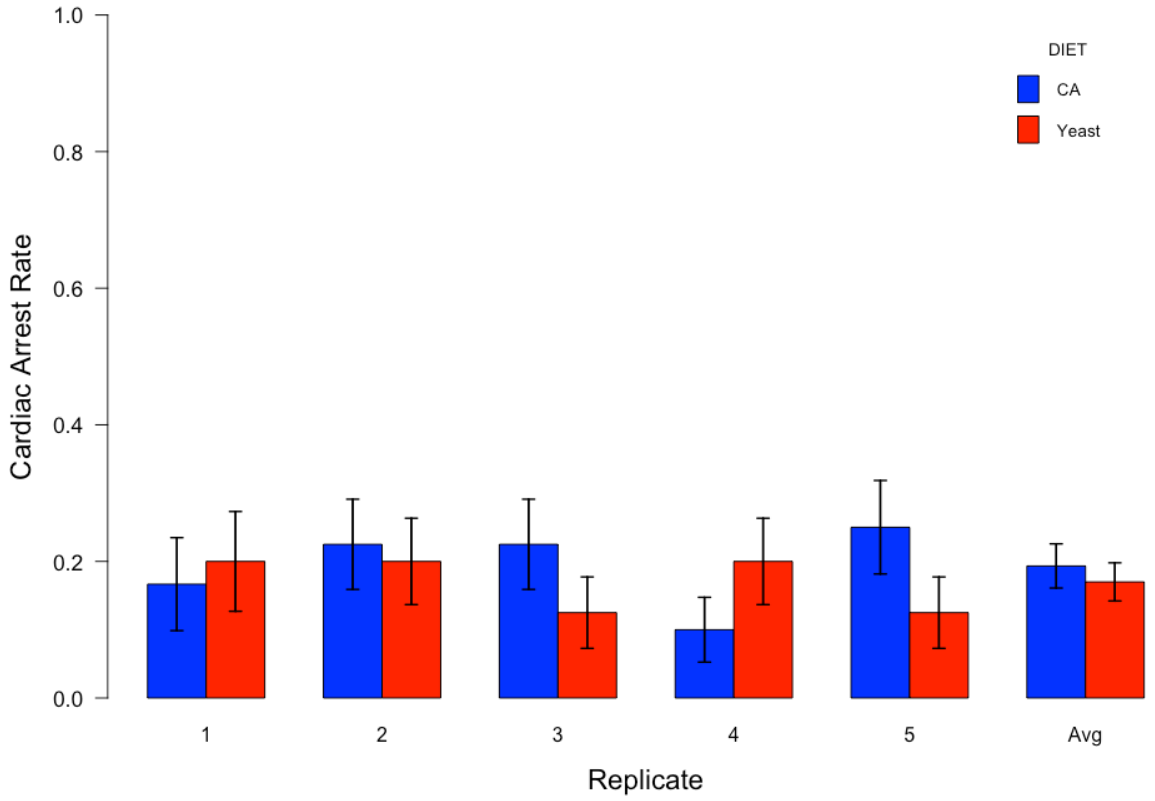

Figure 4.4. The average rate of cardiac arrests of female fruit flies from our $CO_{1\text{-}5}$ populations (mean ± 1 SEM). The populations fed a caffeinated diet (CA) did not have significantly different average rate of cardiac arrests than the CO populations receiving the controlled yeast diet (p-value = 0.596).

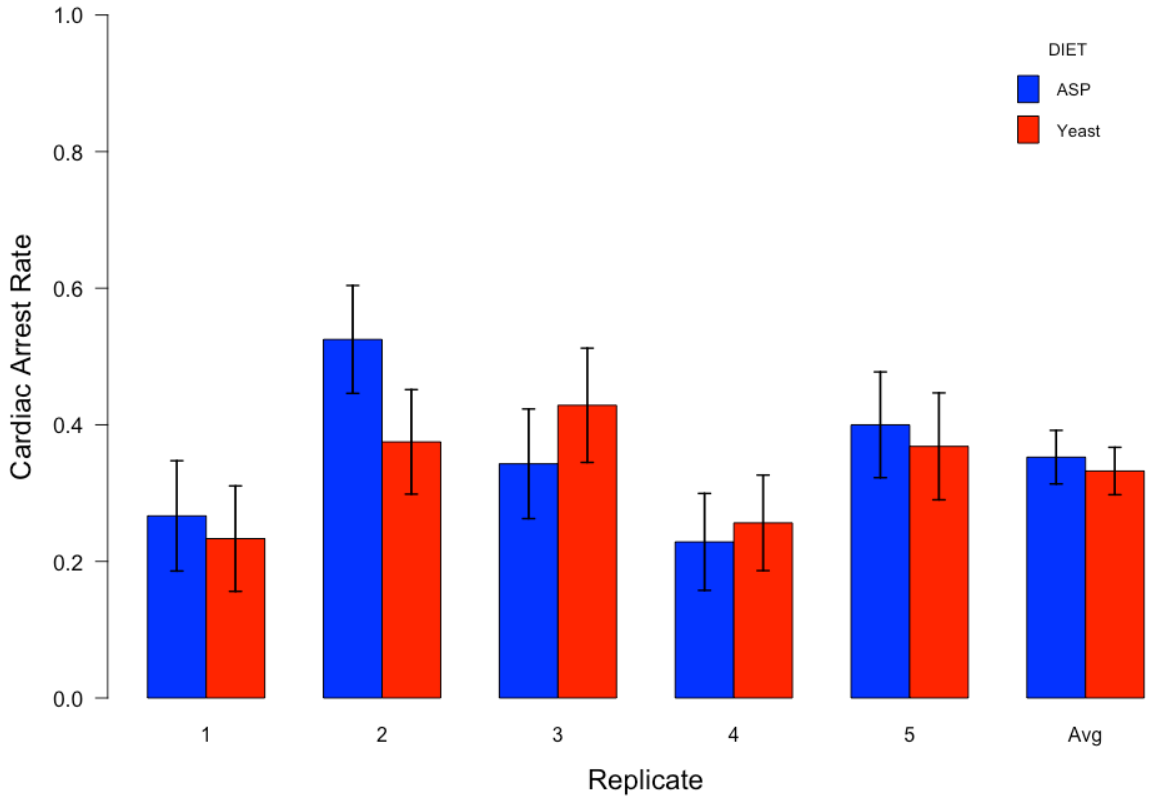

Figure 4.5. The average rate of cardiac arrests of female fruit flies from our $ACO_{1\text{-}5}$ populations (mean ± 1 SEM). The populations fed an aspirin-supplemented diet (ASP) did not have significantly different average rate of cardiac arrests than the ACO populations receiving the controlled yeast diet (p-value = 0.721).

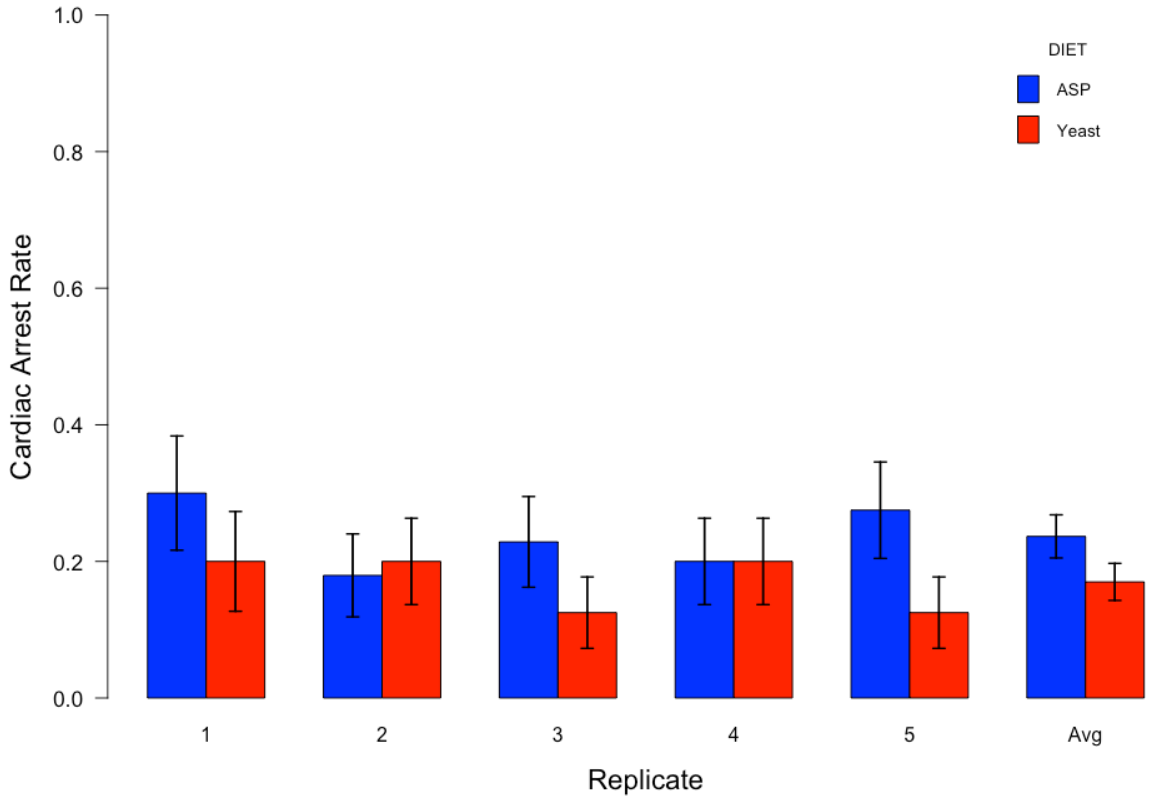

Figure 4.6. The average rate of cardiac arrests of female fruit flies from our $CO_{1\text{-}5}$ populations (mean ± 1 SEM). The populations fed an aspirin-supplemented diet (ASP) did not have significantly different average rate of cardiac arrests than the CO populations receiving the controlled yeast diet (p-value = 0.152).

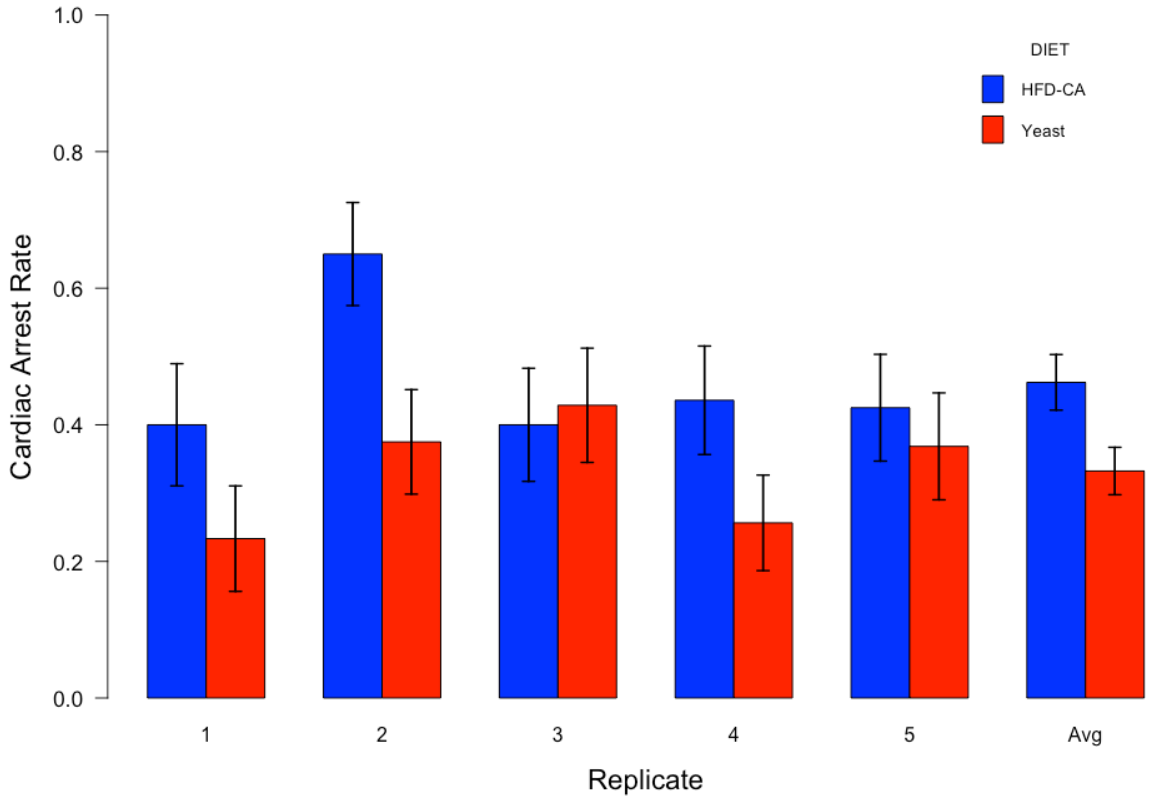

Figure 4.7. The average rate of cardiac arrests of female fruit flies from our $ACO_{1\text{-}5}$ populations (mean ± 1 SEM). The populations fed a high-fat diet supplemented with caffeine (HFD-CA) had higher average rate of cardiac arrests than the ACO populations receiving the controlled yeast diet (p-value = 0.013).

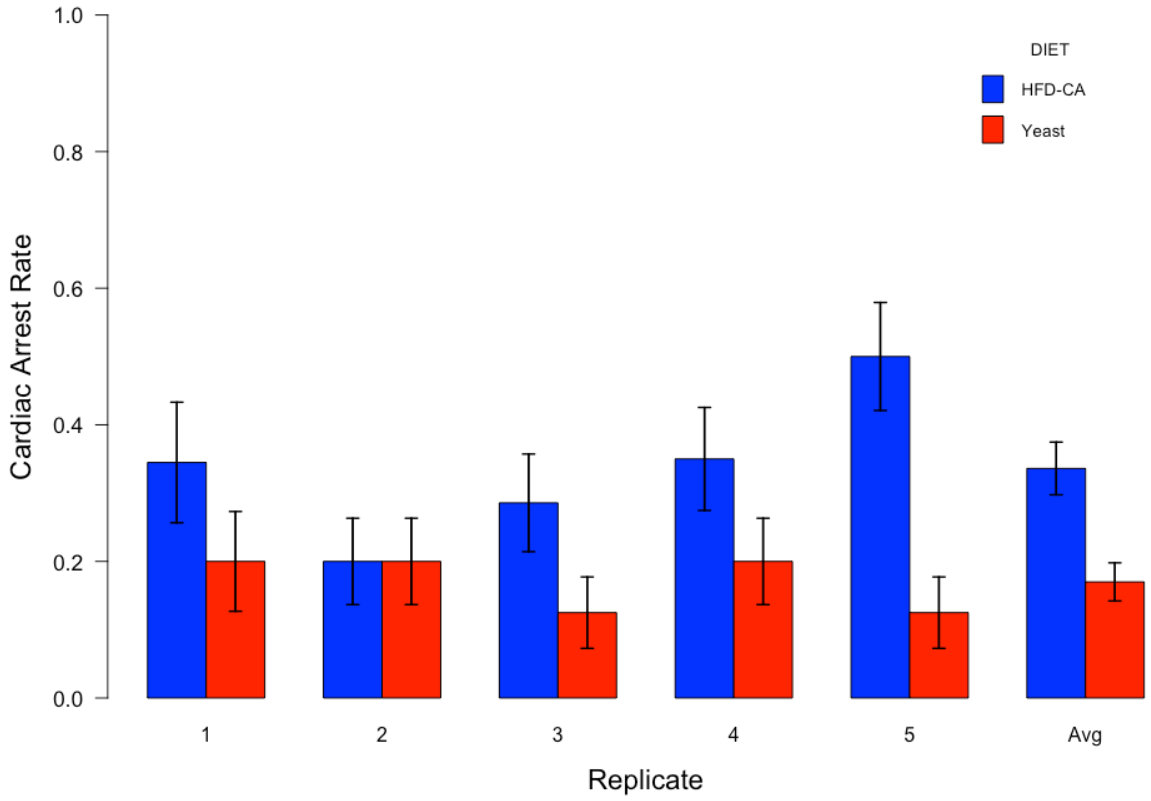

Figure 4.8. The average rate of cardiac arrests of female fruit flies from our $CO_{1\text{-}5}$ populations (mean ± 1 SEM). The populations fed a high-fat diet supplemented with caffeine (HFD-CA) had higher average rate of cardiac arrests than the CO populations receiving the controlled yeast diet (p-value < 0.001).

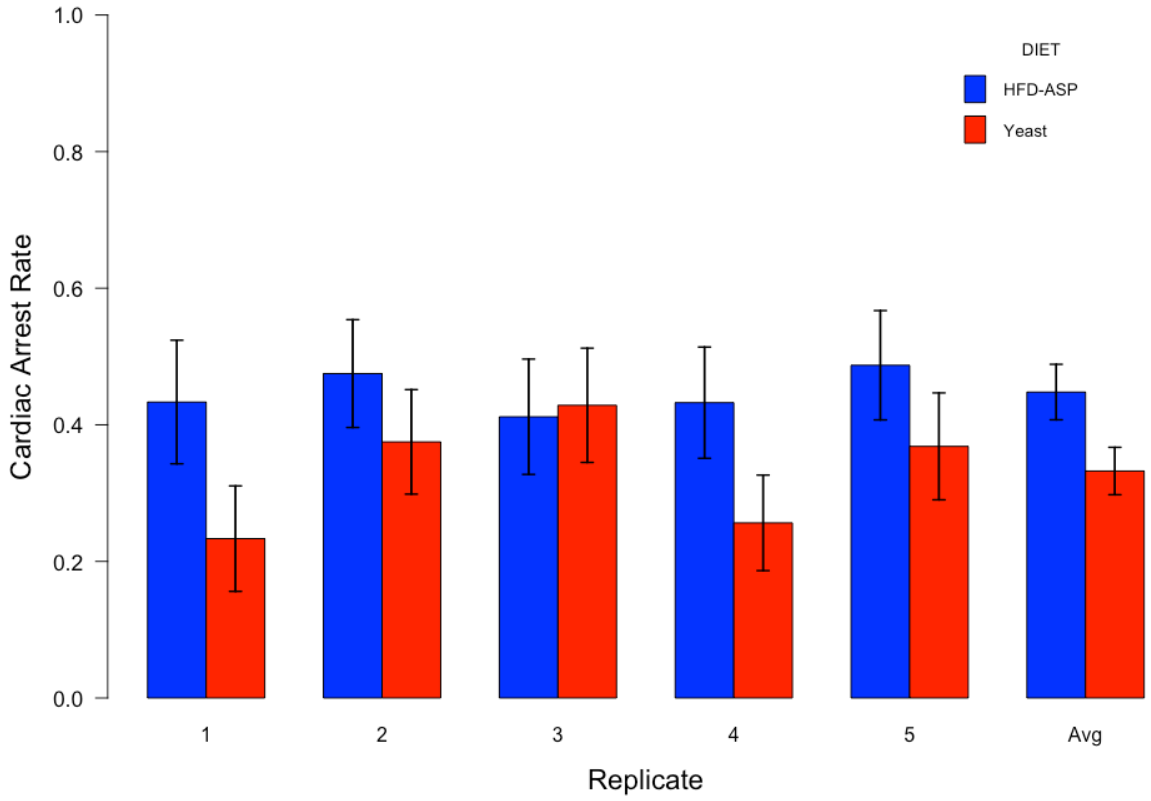

Figure 4.9. The average rate of cardiac arrests of female fruit flies from our $ACO_{1\text{-}5}$ populations (mean ± 1 SEM). The populations fed a high-fat diet supplemented with aspirin (HFD-ASP) had higher average rate of cardiac arrests than the ACO populations receiving the controlled yeast diet (p-value = 0.035).

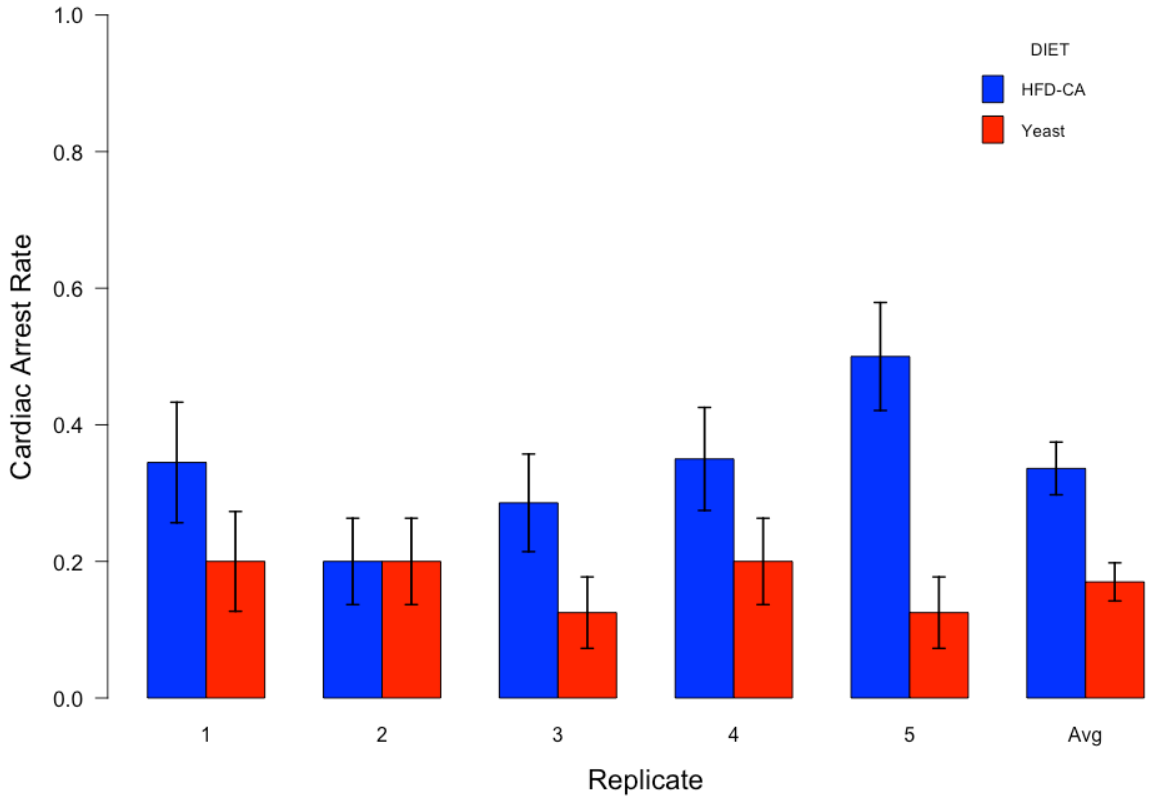

Figure 4.10. The average rate of cardiac arrests of female fruit flies from our $CO_{1\text{-}5}$ populations (mean ± 1 SEM). The populations fed a high-fat diet supplemented with aspirin (HFD-ASP) had higher average rate of cardiac arrests than the CO populations receiving the controlled yeast diet (p-value < 0.001).

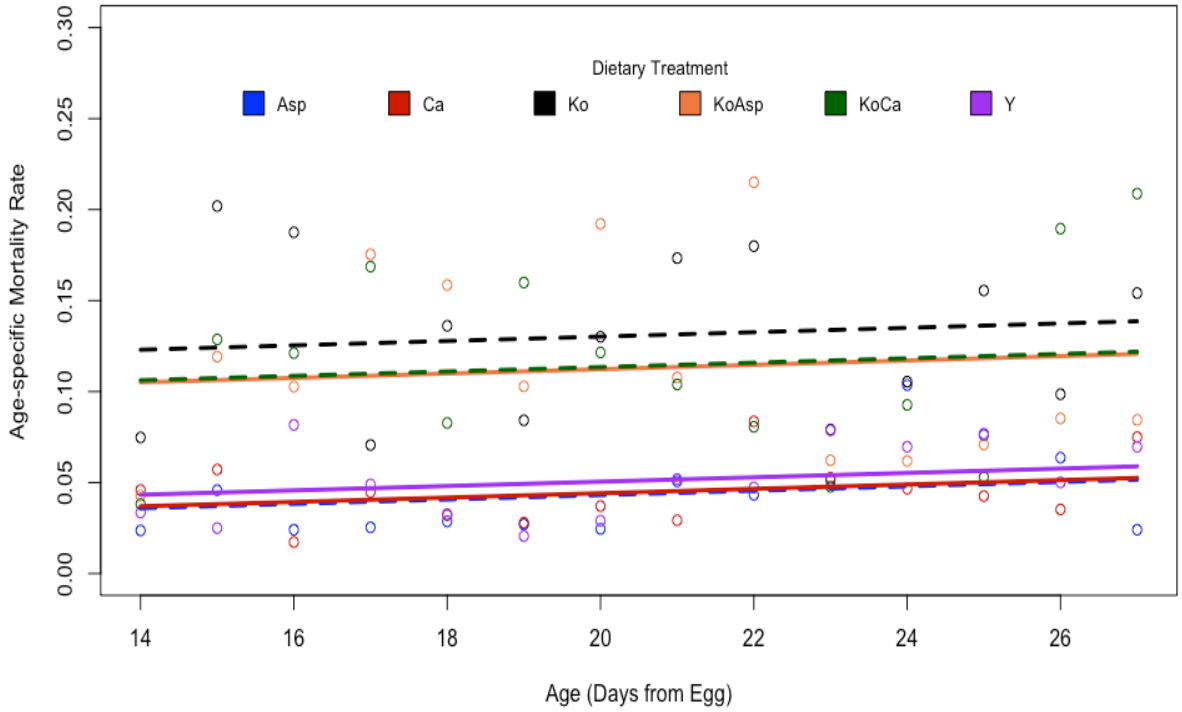

Figure 4.11. The average age-specific mortality rates from ages 14-27 days from egg for female flies from the ACO populations exposed to one of the six dietary treatments. The three diets with a coconut oil component produced significantly higher mortality rates in comparison to the ACO populations exposed to either control yeast diet, caffeine diet, or aspirin diet (p-values < 0.001).

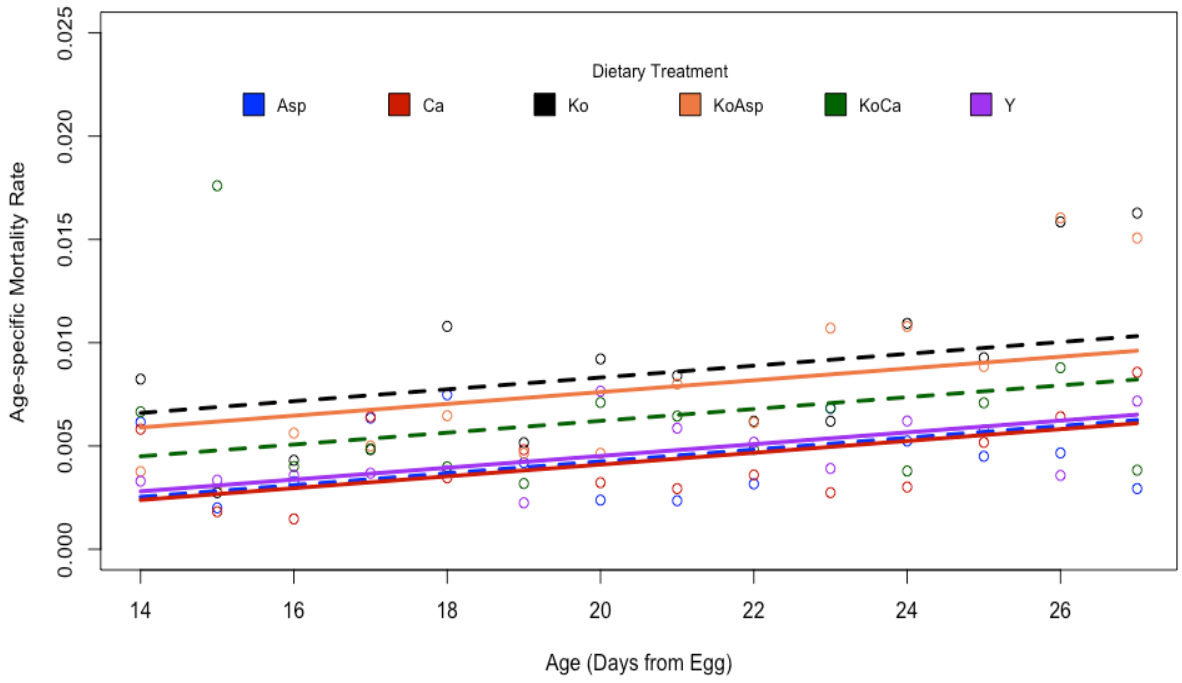

Figure 4.12. The average age-specific mortality rates from ages 14-27 days from egg for female flies from the CO populations exposed to one of the six dietary treatments. The high fat diet (Ko) and high-fat diet supplemented with aspirin (KoAsp) produced significantly higher mortality rates in comparison to the CO populations exposed to either control yeast diet, caffeine diet, or aspirin diet (p-values < 0.05). The high fat diet supplemented with caffeine (KoCa) did not significantly differentiate the mortality rates in comparison to any of the other diets.

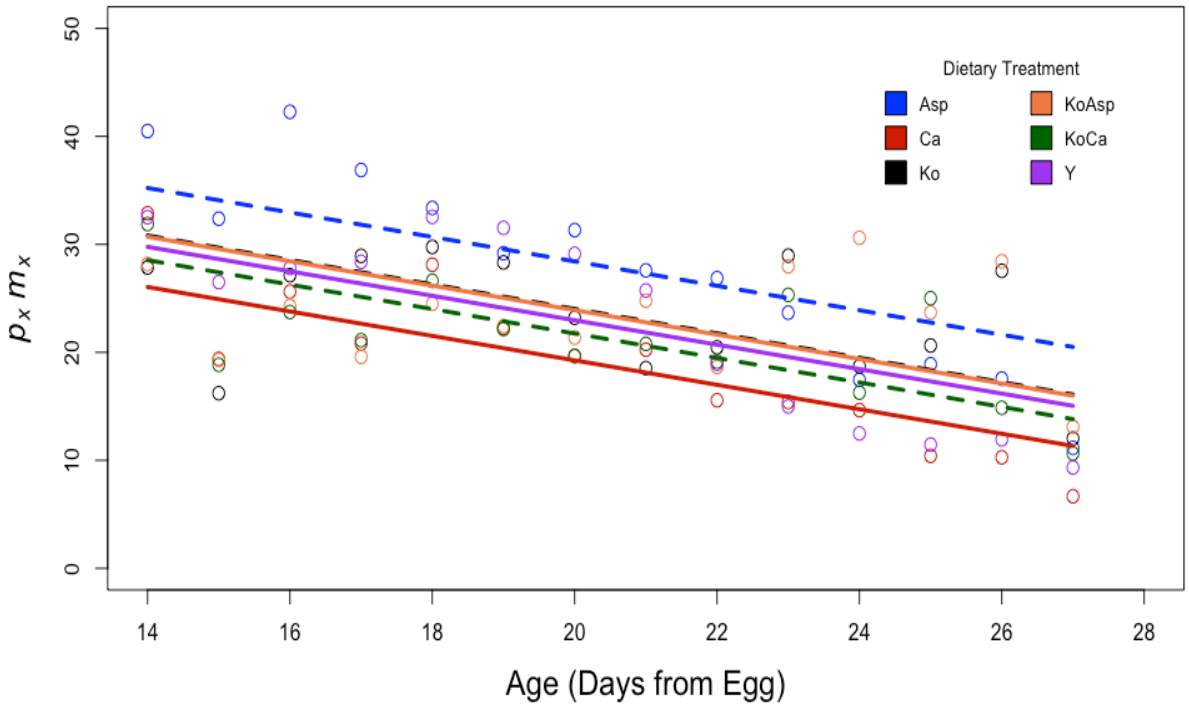

Figure 4.13. The average age-specific $p_x m_x$ of $ACO_{1\text{-}5}$ female flies exposed to one of the six dietary treatments. $ACO_{1\text{-}5}$ populations fed an aspirin-supplemented diet had significantly higher $p_x m_x$ value during this 14 day interval. The aspirin dietary treatment was only treatment to significantly differ from ACO populations exposed to the control yeast diet.

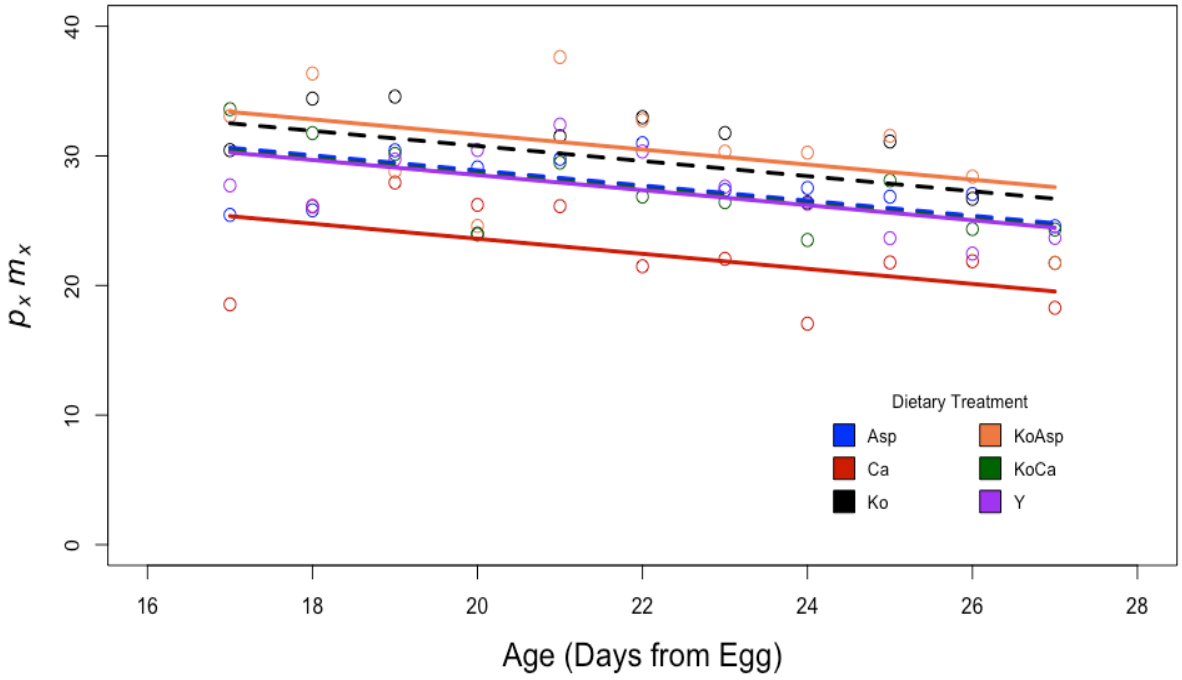

Figure 4.14. The average age-specific $p_x m_x$ of $CO_{1\text{-}5}$ female flies exposed to one of the six dietary treatments. The high-fat diet supplemented with aspirin (KoAsp) was only diet to significantly increase $p_x m_x$ values in $CO_{1\text{-}5}$ populations during this 11 day interval. The caffeine treatment was only treatment to significantly lower the $p_x m_x$ of CO populations compared to CO populations fed the control yeast diet.

CHAPTER 5

CONCLUSION

In conclusion, we would like to bring out the following major scientific themes that this dissertation instantiates and elucidates: (a) the speed of physiological responses to both moderate and intense laboratory selection; (b) the inferential power of combining data from experiments that feature sequential stressors, dietary manipulation, and laboratory selection; and (c) the value of the *Drosophila* model for the study of cardiovascular function.

I. Rapid Physiological Response to Selection

Chapters II and IV are both revealing with respect to speed of evolution among physiological characters undergoing laboratory evolution. In the case of Chapter II, which involved 30 populations that chiefly were subjected to different demographic regimes, we see relatively rapid convergence of physiological characters among populations that share the same distal, or most recent, selection regime. These physiological characters include starvation resistance, desiccation resistance, flight endurance, and heart robustness under pacing. Qualitatively speaking, these physiological characters show the same kind of rapid evolutionary convergence as that shown previously for both life-history characters (Burke et al. 2016) and genome sequences (Graves et al. 2017) among the same 30 populations. In almost every respect, recent selection regime dominates over past evolutionary history for physiological characters, just as does for fitness, life history, and the underlying genomics. This in turn suggests that Darwinian adaptation at every mechanistic level can be powerfully determinative.

From a somewhat different perspective, Chapter IV provides an example of rapid differentiation of key physiological characters under laboratory selection. After as little as 55

generations, the ten populations selected for starvation resistance developed very high levels of starvation resistance: going from about 60 hours of survival on average under conditions of total starvation to about 150 hours. Together with this change in starvation resistance, lipid levels increased from about 0.05 milligrams per fly to about 0.125 milligrams per fly, a comparable increase in magnitude. This is comparable to previous work in our laboratory on the rapid increases in starvation or desiccation resistance that can be produced by directional selection on these characters themselves (e.g. Archer et al. 2003; Archer et al. 2007; Rose et al. 1992).

As discussed also by Burke and Rose (2009), replicated laboratory selection in *Drosophila* is a very powerful paradigm for producing rapid differentiation in physiological characters that then provides useful systems for physiological analysis (cf. Bradley et al. 1999; Rose and Bradley, 1998; various publications collected in Rose et al. 2004). Indeed, this is now becoming a well-established point for other experimental paradigms that have been used within the field of evolutionary physiology, such as high-temperature selection in *Escherichia coli* (e.g. Bennett et al. 1990; Tenaillon et al. 2012) and running-behavior selection in *Mus domesticus* (Garland et al. 2002; Swallow et al. 2009). However, we note that the diversity of modalities of physiological selection and character evolution has been greatest for research on the evolutionary physiology of *D. melanogaster.*

II. Combining Experimental Strategies in Evolutionary Physiology

In the past, our laboratory has conducted research in evolutionary physiology using multiple experimental paradigms. In particular, as just discussed, we have studied physiological characters in the context of experimental evolution, both when those characters were not subjected to focal selection and when they were subjected to such focal selection. In addition, we

and our collaborators have used dietary manipulation with respect to both amount of food and kind of food (e.g. Chippindale et al. 1993; Chippindale et al. 1997; Jafari et al. 2007; Matsagas et al. 2009).

In Chapter V of this thesis, we have continued our previous on dietary manipulation, varying both nutrients and pharmacological substances: caffeine, coconut oil, and aspirin. But in Chapter III, we add a second type of physiological manipulation, specifically sequential combinations of stressors: flight then starvation; flight then desiccation; flight then electrical pacing; starvation then flight; starvation then electrical pacing; desiccation then flight; and desiccation then electrical pacing.

The interesting thing about this combination of experimental paradigms employed on one system, is that it enables us to refine our interpretations of the physiological machinery of the fruit fly. To give what is, for us at least, the most intriguing insight underscored by all of this work put together, high levels of lipid evidently impair heart robustness. This is illustrated by the impact of starvation on the response of the fly heart to electrical pacing. Even though moderate levels of starvation constitute a physiological stress, interestingly such starvation enhances the robustness of the fly heart under electrical pacing, as shown in Chapter III. Conversely, selecting for high levels of starvation resistance increases lipid levels and decreases heart robustness, as shown in Chapter IV. Finally, diets that are high in fats lead to decreased heart robustness, as shown in Chapter V. Together, these findings from three quite different experimental paradigms all support the general physiological inference that very high levels of lipid present challenges for the *Drosophila* heart, such that interventions which increase lipid have adverse effects on heart function, while interventions that decrease lipid benefit heart function. However, it should be noted that flies selected for later reproduction have increases in rates of adult survival (e.g.

Rose et al. 2002), increased levels of lipid (Djawdan et al. 1998), but increased heart robustness (Shahrestani et al. 2017). Thus lipid levels are not the only factor impinging on heart robustness.

III. What We Have Learned about Hearts from *Drosophila*

The foregoing discussion illustrates one of the most important findings of research on the *Drosophila* heart: high levels of lipid appear to damage it. This appears to occur whether the increased levels of lipid are due to heredity (Chapters II, IV) or diet (Chapter V), a finding that other fly heart labs have found as well (e.g. Birse et al. 2010; Diop and Bodmer 2012; Diop et al. 2015; Hardy et al. 2015). This pattern is not the only feature of the evolutionary physiology of the fly heart, as just mentioned, in that slightly fatter flies can live longer and have more robust hearts (Shahrestani et al. 2017). But such flies could have evolved compensatory physiological mechanisms that enable them to combine elevated lipid levels with improved heart robustness, though we have no evidence for that at present.

Looking upward from the heart to fly life history, the results of Chapters II and IV show the importance of heart function for fly survival and reproduction. As demographic selection regimes favor increased adult survival and later reproduction, they systematically tune up heart function, as shown in Chapter II. On the other hand, as lipid levels increase with selection for starvation resistance, both heart function and adult survival are reduced, as shown in Chapter IV. Shahrestani et al. (2017) showed directly that electrical pacing reduces both heart function and subsequent survival among individual flies. This doesn't definitively establish a direct connection between heart function and adult survival, but it does support the hypothesis that they are indeed so connected.

We expect that continuing research on the fly heart will provide more insights into its underlying physiology. We also expect that the connections among heart function and other physiological characters will be delineated further. Finally, we hope that insights derived from the use of the multiplicity of research paradigms now being employed in *Drosophila* heart research will help increase our understanding of heart disease and heart health in human populations, the species of greatest everyday interest for most people.

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