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The Effects of Starvation Selection on Drosophila Melanogaster Life History and Development

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THE EFFECTS OF STARVATION SELECTION ON *DROSOPHILA MELANOGASTER* LIFE HISTORY AND DEVELOPMENT

by

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ABSTRACT

The Effects of Starvation Selection on
Drosophila melanogaster
Life History and
Development

by

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In nature, animals may endure periods of famine to complete their life cycles. Starvation stress will increase in populations as climates around the world change. To predict how populations may respond to such a stress, laboratory experimentation becomes essential. The evolutionary process of adaptation, its innovations and their trade-offs, can be studied in populations experiencing starvation stress. For this purpose outbred populations of *Drosophila melanogaster* were selected for starvation resistance in the laboratory.

After 60+ generations of starvation selection the starvation-selected flies have gone from surviving 2-3 days without food to 12-14 days without food. How this amazing feat of resistance is achieved in these flies is the subject of this dissertation. *Drosophila* have three mechanisms for increasing their starvation resistance. 1) Increase energy reserves, 2) decrease rate of energy use, and 3) require less energy to maintain life. Here I examined each of these strategies in the starvation-selected flies. Starvation-selected flies store nearly 3 times the amount of lipids considered normal and use those lipids at a slower rate by having lowered their metabolic rate. These findings support the

use of mechanisms 1 and 2 to survive starvation stress; however no evidence supporting mechanism 3 was discovered. The lipids, so important for surviving starvation, were found to be accumulated during larval development. The storage of such large amounts of lipids may also be causing a trade-off between storage of different energetic nutrients.

Acquiring starvation resistance has affected other life history traits negatively. Fecundity is low in starvation-selected flies, and egg-to-puparium development is extended by at least 24 hours, decreasing the overall fitness of the starvation-selected fly populations. This extension in development is of particular interest, because the lipid stores used to resist starvation are accumulated during larval development; an extension in development may contribute to extra lipid stores. The delay in larval development is most likely due to a delay in the hormonal cascade responsible for regulating development. Larval development time was shortened significantly in flies fed 20-hydroxyecdysone (20E) early, but lipid content was only reduced by a small amount in the starvation-selected flies. Development time therefore contributes to lipid stores to some extent, but lipid metabolism during development must also play a significant role.

The delay in the hormonal cascade responsible for regulating development, and no change in the rate of mass accumulation, in combination are consistent with a model developed in *Manduca sexta* that selection for starvation resistance is positively selecting for longer development time and larger body size. This evolutionary model may have promise as a model for studying and predicting evolutionary mechanisms in drosophila as well.

Overall the starvation-selected flies provide an excellent model for investigating starvation resistant mechanisms and how they evolved under selection. Energy storage

predominates these mechanisms at the expense of changes in development. The findings brought together here contribute significantly to our understanding of the physiological mechanisms behind starvation resistance and contribute to developing models to predict the evolutionary outcome of starvation stress.

ACKNOWLEDGMENTS

I learned rather quickly upon beginning graduate school that there is no such thing as a completely independent PhD project. As a graduate student I was reliant on the goodwill and kindness of my other graduate student peers and many faculty mentors. Without their help, guidance, and effort I would never have been able to finish. Thank you so very much for believing in me and investing your time in my future.

Outside of the university one also must have support. I have been very fortunate to have a very loving and proud family. Every step of the way they have done nothing but provide their unwavering support in all my efforts. If it was within their power to help me, they never hesitated. Knowing that one has such love and encouragement behind them can make all the difference. Thank you Mom & Dad & Shawn my brother & Duncan my husband.

My biggest thanks of all must be reserved for the man who saw potential in me to be a great graduate student even when no one else did. Thank you Dr. Allen Gibbs for providing me with the courage to continue and then having the patience to put up with me when I did.

DEDICATION

To my cat, Spookie,

who slept, begged for tuna, and

played with her toy mouse

while I wrote it.

She reminded me of the

most important things in life.

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Previously published as:
Gibbs, A. G., & Reynolds, L. A. (2012). *Drosophila* as a model for starvation: evolution, physiology, and genetics. In M. D. McCue (Ed.), *Comparative physiology of fasting, starvation, and food limitation* (pp. 37-54). New York: Springer.

1.1 Introduction

Drosophila melanogaster is one of the primary genetic models for understanding how nutritional limitation affects cellular physiology, because many of the molecular and cellular signaling pathways are shared among invertebrates and vertebrates. To a lesser extent, it is a model for organismal responses, although differences in endocrine systems sometimes make the link to vertebrates one of analogy rather than homology. *Drosophila* is also an excellent model for the evolution of starvation responses. The evolutionary history of the genus has been well studied, and *D. melanogaster's* short generation time and ease of maintenance have allowed experimental evolution studies on starvation resistance. We review here studies of starvation in *Drosophila* at multiple levels of organization, from species to molecules. A great advantage of *Drosophila* is the ability to traverse these levels relatively easily, and information across all levels is now being integrated in many labs around the world.

It is important to recognize at the outset that *D. melanogaster* is only a *model* for other species, including other *Drosophila* species. We were charged with reviewing the physiology of starvation specifically in *Drosophila,* and so we do not refer to the large

and interesting body of related work done with *Manduca, Locusta, Bombyx* and a wide variety of other insects. The literature on *Drosophila* alone is extensive – our recent Web of Science search for "drosophila and feeding" returned nearly 2000 citations. This review will therefore necessarily skim the surface and omit a great deal of interesting information about starvation in *Drosophila*.

1.2 Starvation Resistance in Natural Populations

The role of starvation stress in the ecology of *Drosophila* species is very poorly understood. (The ecology of *Drosophila* in general is poorly understood.) It is clear, however, that *Drosophila* species vary greatly in their ability to survive starvation stress. van Herrewege and David (1997) found that *Drosophila* species differed up to five fold in their survival in humid air. Starvation resistance was highly temperature dependent, with flies surviving approximately twice as long at 17^{o}C as at 25^{o}C. Species from temperate regions tended to survive longer than tropical species. The temperate species studied also tended to be larger, which may have contributed to longer survival times (Figure 1.1). On the other hand, flies from temperate populations of two species were larger than tropical congeners, but did not differ much in starvation resistance.

Many *Drosophila* species have broad geographic ranges, allowing intraspecific studies of local adaptation in starvation resistance. The Indian subcontinent has been particularly well studied. Northern populations of several species have lower starvation resistance compared to southern, subtropical populations (Parkash & Munjal, 2000; Parkash, Sharma, & Sharma, 1994; Sisodia & Singh, 2010). Starvation resistance also

Figure 1.1

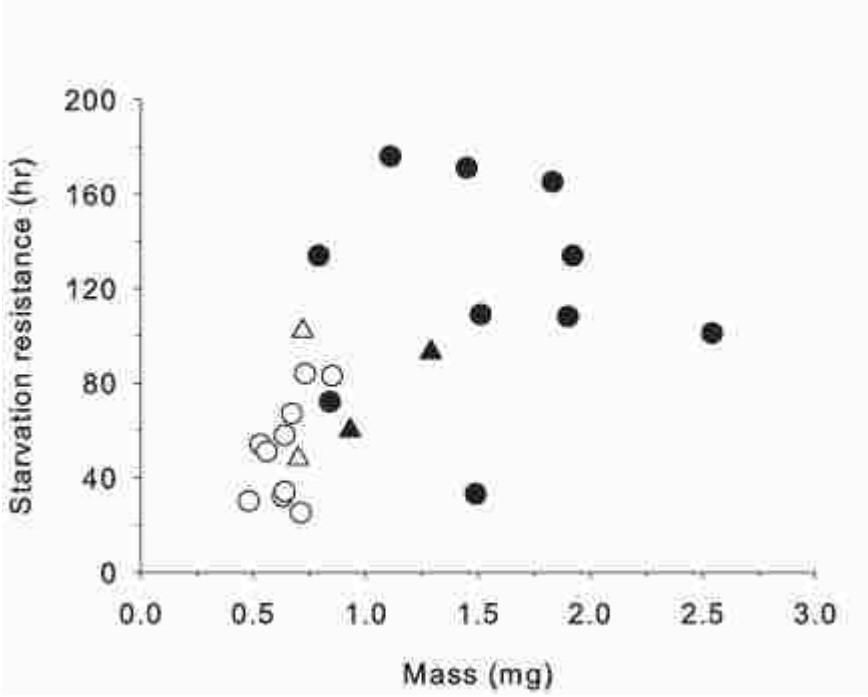


Figure 1.1 Starvation resistance of 22 species of *Drosophila:* Male flies were assayed at 25^{o}C. Open circles, tropical species; filled circles, temperate species. Triangles indicate tropical and temperate populations of *D. melanogaster* and *D. simulans*. Data from van Herrewege and David (1997).

increases with latitude in Australian populations of *D. birchii* (Griffiths, Schiffer, & Hoffmann, 2005).

In eastern North America, an opposing latitudinal cline occurs. Populations of *D. melanogaster* in the north are more starvation resistant than southern populations (Schmidt, Matzkin, Ippolito, & Eanes, 2005; Schmidt & Paaby, 2008). Robinson et. al. (2000) also found no correlation between latitude and starvation resistance in *D. melanogaster* from South America. In Australia, differences in starvation resistance between populations of *D. melanogaster* were found, but these were not correlated with environmental conditions (Hoffmann, Hallas, Sinclair, & Mitrovski, 2001; Hoffmann, Shirriffs, & Scott, 2005; Hoffmann & Weeks, 2007), whereas Philippine *Drosophila* species varied within, but not among, populations (van der Linde & Sevenster, 2006).

The explanation(s) for differing geographic patterns in starvation resistance are not clear. Parkash and Munjal (2000) argue that tropical populations are more susceptible to starvation because of higher metabolic rates related to high habitat temperatures. In North America, northern populations of *D. melanogaster* are more likely to undergo reproductive diapause under simulated winter conditions (Schmidt et al., 2005). Schmidt & Paaby (2008) concluded that females able to use reproductive diapause to overwinter are more resistant to stress in general, including starvation. Australian populations also differ in reproductive patterns in the winter (Hoffmann, Scott, Partridge, & Hallas, 2003; Mitrovski & Hoffmann, 2001), suggesting a potential link between reproduction and stress resistance.

It should also be noted that the latitudinal ranges for these studies differ. For example, the northernmost Indian populations studied were from similar latitudes to the

southernmost North American populations. Differing types of selection at the extreme latitudes could result in higher starvation resistance in both regions. For example, global scale atmospheric circulation patterns (Hadley cells) create generally lower humidity approximately 30^{o} north and south of the equator. Natural selection for surviving desiccation could trade off against starvation resistance (Parkash, Aggarwal, & Kalra, 2012; Parkash & Munjal, 2000; Parkash et al., 1994).

An alternative to comparative studies of starvation resistance is to study its evolution in the laboratory. *Drosophila melanogaster* is a widely used experimental model for the evolution of stress resistance (Garland & Rose, 2009). The use of replicated populations (and unselected control populations) under controlled conditions allows correlations and tradeoffs between traits to be assessed and tested in a rigorous manner, although laboratory environments are not necessarily as simple as they appear (Gibbs & Gefen, 2009). Starvation resistance evolves rapidly when populations are subjected to strong selection each generation (Rose, Vu, Park, & Graves, 1992). Selection on a poor diet (lemons) also results in increased starvation resistance (Harshman, Hoffmann, & Clark, 1999). Most studies have involved selection for adult starvation resistance, but at least one study on larval selection has been performed (Kolss, Vijendravarma, Schwaller, & Kawecki, 2009).

1.3 Physiological Mechanisms of Starvation Resistance

At the organismal level, there are three mechanisms by which starvation resistance can be increased, as illustrated in Figure 1.2. Animals can store more energy (lipids, carbohydrates, protein), they can consume it at a slower rate, or they can tolerate

Figure 1.2

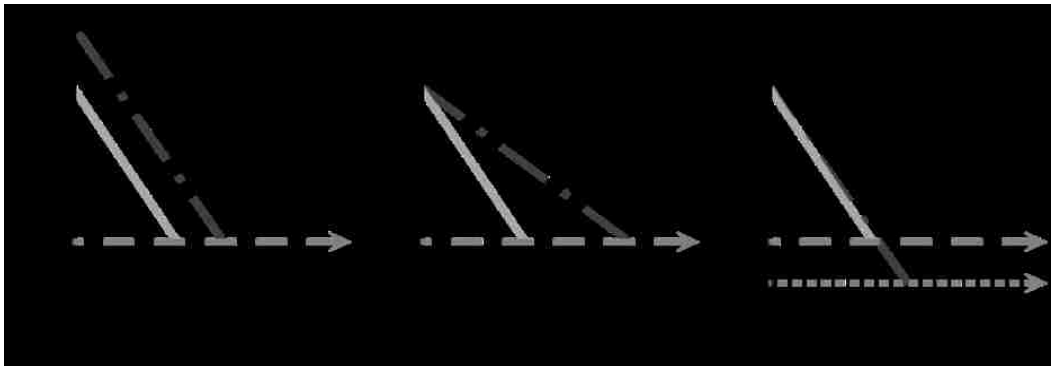


Figure 1.2 Potential organismal mechanisms to increase starvation resistance: A. Increased energy storage. B. Reduced energy consumption. C. Lower energetic threshold for mortality.

loss of a greater fraction of their initial energy supply. These mechanisms are not mutually exclusive. A fourth, behavioral strategy is cannibalism. When flies are starved in groups, in principle the longest survivors can consume those which have already died. This is not seen in wildtype flies (Huey, Suess, Hamilton, & Gilchrist, 2004), but could evolve in starvation-selected populations.

Starvation resistance is positively correlated with lipid content among different *Drosophila* species (Bharathi, Prasad, Shakarad, & Joshi, 2003; van Herrewege & David, 1997). In fact, the differences between tropical and temperate species seen in Figure 1.1 are largely due to higher relative lipid content. Similar correlations between lipid content and starvation resistance occur within species (Ballard, Melvin, & Simpson, 2008; Parkash, Tyagi, Sharma, & Rajpurohit, 2005; Sisodia & Singh, 2010). Greatly increased lipid storage is a consistent finding in starvation selection experiments (Chippindale, Chu, & Rose, 1996; Djawdan, Rose, & Bradley, 1997; Harshman, Hoffmann et al., 1999; Schwasinger-Schmidt, Kachman, & Harshman, 2012). Lipid contents are generally much higher than in natural populations, suggesting that lipid storage has an evolutionary cost. Carbohydrates have received far less attention than lipids as energy stores, but also increase under starvation selection (Djawdan et al., 1997). Thus, energy storage, particularly in the form of lipids, is a consistent marker for starvation resistance.

The relationship between metabolic rates and starvation resistance is murkier. Surprisingly, no systematic comparative studies of metabolic rates in natural populations of *Drosophila* appear to have been done, at least not in the context of starvation stress. Metabolic rates differ substantially among species (Gibbs, Fukuzato, & Matzkin, 2003; Marron, Markow, Kain, & Gibbs, 2003). Some of this variation may be related to water

conservation, as desert (cactophilic) *Drosophila* have lower metabolic rates than other species after correction for body size and phylogenetic relationships (Gibbs et al., 2003). Tolerance of low energy content has not been studied (Rion & Kawecki, 2007).

In starvation selection experiments, the evidence for evolution of reduced metabolism is mixed. Starvation-selected flies often have lower mass-specific metabolic rates than controls (Djawdan et al., 1997; Harshman, Hoffmann et al., 1999). However, they are also larger because of their greater energy stores; when this is taken into consideration metabolic differences may disappear (Djawdan et al., 1997). Baldal et al. (2006) found that starvation-selected females actually tended to have higher metabolic rates than controls in the absence of food. No differences were seen when food was present, but metabolic rates consistently are lower when flies are starved than when they are fed (Baldal, Brakefield, & Zwaan, 2006; Djawdan et al., 1997). Harshman and Schmid (1998) also found no relationship between metabolic rates and starvation resistance. More recently, Schwasinger-Schmidt et al. (2012) found some support for the idea that starvation-selected flies are less active, and therefore should have lower metabolic rates. In summary, lower metabolic rates may contribute to increased starvation resistance in *Drosophila*, but their contribution is inconsistent and is certainly less significant than differences in energy storage.

1.4 Starvation and Life History Traits

A fundamental tradeoff in life history evolution exists between allocation of resources to survival and reproduction. This tradeoff can be alleviated by acquiring more resources (de Jong, 1993), as exemplified by lipid accumulation in starvation-selected populations of *Drosophila*. Resource acquisition may have its own costs, however.

Starvation-selected flies take longer to develop (Chippindale et al., 1996; Harshman, Hoffmann et al., 1999) and have lower fecundity than controls (Kolss et al., 2009; Wayne, Soundararajan, & Harshman, 2006). This is despite their larger body size and higher lipid content, factors that are generally correlated with higher fecundity in insects.

This conundrum may be explained by the complex life cycle of *Drosophila*. Holometabolous insects have striking differences in life history from vertebrates. In the case of *D. melanogaster*, eggs hatch into a larva that is essentially a feeding and growth machine. Over 3 days, the larva increases in mass by approximately 200-fold (Church & Robertson, 1966). Soon thereafter it enters a 15-24 hour wandering phase, during which it ceases feeding, leaves the media, and searches for a pupation site. The larva selects a spot, secretes a glue protein that adheres the animal to the substrate, and undergoes metamorphosis. Approximately 4 days later, an adult fly emerges from the pupal case. The adult feeds and allocates resources between somatic maintenance and reproduction. Thus, the life history of *Drosophila* can be broadly separated into 3 nutritional states: a feeding and growth stage, a non-feeding period lasting from late larval through early adult development, and a feeding but non-growing adult stage.

Drosophila pupae consume less than half of their stored lipids during metamorphosis, so flies eclose to adulthood with an energetic reserve (Merkey, 2011). Starvation-selected adults eclose with greater lipid stores than unselected controls, so that differences in energy storage occur before adulthood as well as in the young adult (Chippindale et al., 1996). This may be achieved by higher larval feeding rates to grow faster, extending the larval feeding period, consuming less energy during metamorphosis, or some combination of these. Pre-adult stages of starvation-selected lines have not been

well characterized, but selected lines do have longer egg-to-adult development times, suggesting a longer feeding period (Chippindale et al., 1996). Within these populations, individuals with longer development times also survived starvation longer.

Larvae store energy in the larval fat body. The fat body is unique to insects and serves many functions in addition to energy storage, including but not limited to immune responses, detoxification, and endocrine secretion (Hoshizaki, 2005). In comparison to other larval tissues, larval fat body is unusual in that its cells remain intact during metamorphosis and are present in the young adult(Nelliot, Bond, & Hoshizaki, 2006). Most larval tissues undergo programmed cell death in the pupa, with their contents being used to support proliferation of the imaginal disk cells that will form the adult tissues. Larval fat cells escape this fate, then undergo programmed cell death in the first 48 hours of adult life (Aguila, Suszko, Gibbs, & Hoshizaki, 2007). Nutrients released at this time are used to support adult tissues and reproduction (Min, Flatt, Kulaots, & Tatar, 2007; O'Brien, Min, Larsen, & Tatar, 2008).

Recent evidence suggests that the larval fat body has an important role in starvation resistance in young adult flies. Aguila et al. (2007) observed that newly-eclosed female adults survived starvation stress over twice as long as 3-10 day old females. The authors then used a genetic manipulation to delay death of the larval fat cells by approximately two days. These females survived starvation ~24 hours longer than unmanipulated flies (Figure 1.3). These flies also had lower fecundity, suggesting that larval resources are also important for reproduction (Aguila, Hoshizaki and Gibbs, unpublished observations).

Figure 1.3

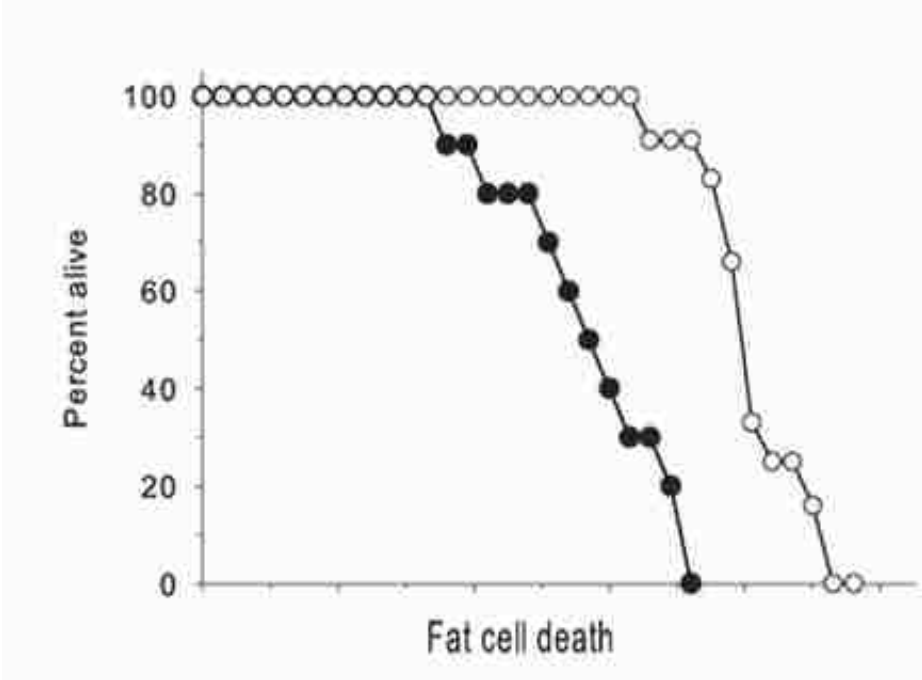


Figure 1.3 Inhibition of programmed fat cell death: increases starvation resistance in *D. melanogaster*. Filled symbols, control flies; open symbols, flies in which fat cell death was inhibited by expression of *diap* (*Drosophila* inhibitor of apoptosis) in the larval fat body. Data from Aguila et al. (2007).

Together these findings suggest that starvation selection affects the physiology of the larval fat body. Increased lipid storage during the larval stage is certainly consistent with this idea. Because all cell division in this tissue occurs embryonically (Hoshizaki, 2005), this probably reflects more lipid per cell rather than more fat cells. Starvation-selected females also have lower early-adult fecundity than controls, despite having more ovarioles (Wayne et al., 2006). Preliminary evidence suggests that fat cell death is delayed in starvation-selected populations (Reynolds and Gibbs, unpublished data), which would cause lower fecundity. The onset of the wandering stage and developmental events in the fat body are regulated by the steroid hormone, 20-hydroxyecdysone (20E); (Bond et al., 2011; Hoshizaki, 2005; Riddiford & Truman, 1993; Rusten et al., 2004). The hormonal basis for fat body changes in all stages of starvation-selected flies is unknown, but 20E signaling is likely to be involved.

1.5 Metabolic Responses to Starvation Stress

Drosophila melanogaster is a widely studied model for starvation responses, but the vast majority of studies have used the third and last larval instar. In adults, food deprivation causes increased activity (Connolly, 1966; Farhadian, Suarez-Farinas, Cho, Pellegrino, & Vosshall, 2012; Knoppien, van der Pers, & van Delden, 2000). Increased energy consumption would appear counterintuitive, but in nature waiting for the next rotting banana to appear makes no sense. Laboratory-selected flies do not have the option of finding a new food source, so they reduce their activity when food is absent (Williams, Rose, & Bradley, 2004). When food is returned, flies increase their feeding rate and allow

more food to accumulate in their crop relative to unstarved controls (Farhadian et al., 2012).

The primary fuel consumed during starvation stress is lipid (Marron et al., 2003), by mechanisms closely resembling, and sometimes homologous to, mammalian regulation of lipolysis (Arrese & Soulages, 2010). Neurosecretory cells in the ring gland secrete adipokinetic hormone (AKH), which activates lipolysis via G protein-mediated phosphorylation of one of the primary proteins associated with lipid droplets in the fat body, lipid storage droplet protein-1 (LSD1), a member of the perilipin protein family. As starvation progresses, transcription of *brummer* (*bmm*) is activated (Groenke et al., 2007). Brummer is the *Drosophila* homolog of adipose triglyceride lipase (Groenke et al., 2005). Lipids are transported in the hemolymph bound to lipophorins, probably in the form of diacylglycerides, rather than triacylglycerides (Canavoso, Jouni, Karnas, Pennington, & Wells, 2001). Oenocytes, specialized cells attached to the inner surface of the animal, take up some of these lipids and store them in a manner analogous to mammalian hepatocytes (Gutierrez, Wiggins, Fielding, & Gould, 2006). Most lipids, however, presumably are absorbed and metabolized by cells throughout the body.

In addition to AKH signaling, the insulin signaling pathway regulates nutrient uptake, storage, and metabolism. This pathway is well conserved between flies and mammals, making *Drosophila* an excellent model for mammals (Figure 1.4). *Drosophila melanogaster* has 7 insulin-like peptides (dILPs) that are homologous to the insulin family in vertebrates, as well as a homologous insulin receptor. The dILPs are expressed at different times by different tissues, but there are some overlapping functions. The most important in terms of nutritional status are dILPs expressed by 7 neurosecretory cells

Figure 1.4

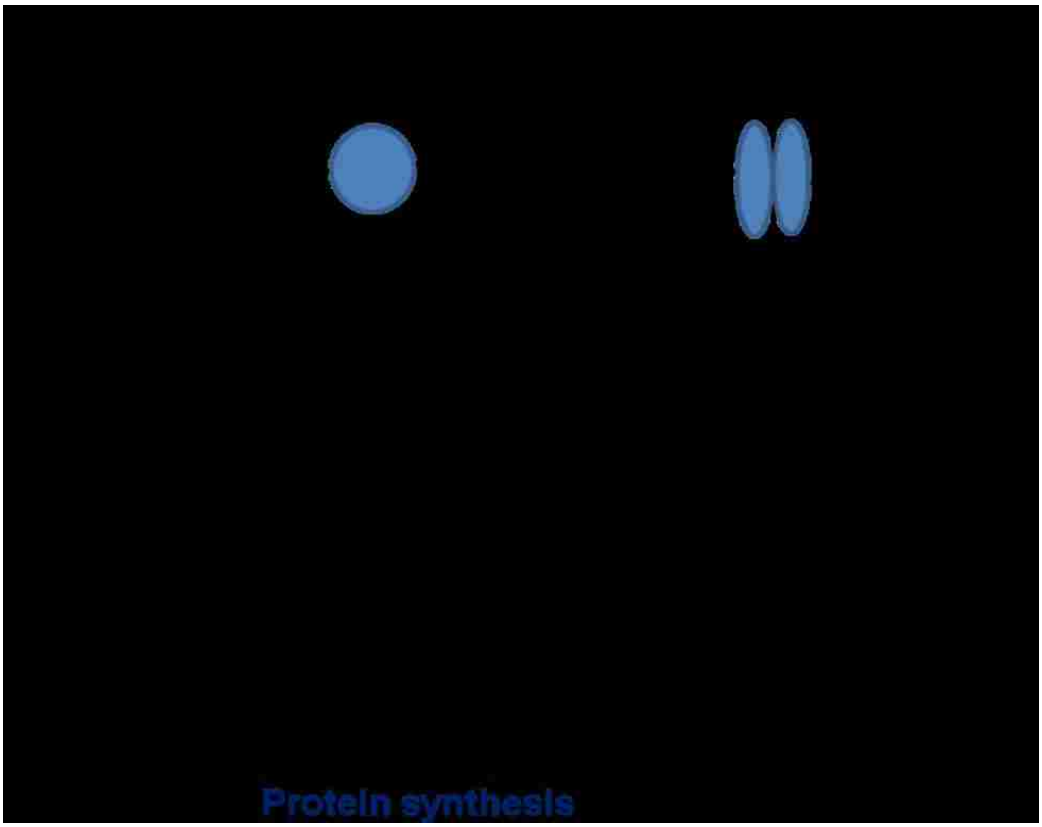


Figure 1.4 Insulin/TOR signaling in *Drosophila*: Only members of these pathways mentioned in the text are shown. Arrows indicate activation of the downstream component; blocked lines indicate inhibition. Dashed lines indicate an indirect effect mediated by one or more intermediate steps. A more complete diagram can be found in Teleman (2010).

(NSCs) in the brain. Ablation of these cells in larvae or adults results in elevated hemolymph trehalose and excess lipid accumulation, analogous to the condition in diabetic mammals (Belgacem & Martin, 2006). However, release of dILPs is not dependent on lipid or carbohydrate levels; instead it depends on an amino acid sensing mechanism in the fat body (Geminard, Rulifson, & Leopold, 2009).

Drosophila have only one insulin receptor (InR), which can bind all 7 dILPs. Binding activates an intracellular signaling pathway strongly resembling, but less redundant than, mammalian insulin signaling (Teleman, 2010). Events include activation of PI3 kinase (PI3K), followed by the protein kinase Akt. Akt then phosphorylates a variety of proteins, including dFOXO, the single *Drosophila* member of the FOXO family of transcription factors. dFOXO regulates transcription of numerous targets (Teleman, Hietakangas, Sayadian, & Cohen, 2008), including 4E-binding protein (4E-BP, or Thor, a general inhibitor of translation). Phosphorylation of dFOXO decreases *Thor* expression, allowing greater protein synthesis.

Akt also indirectly regulates TOR (Target of Rapamycin), a central regulator of cellular metabolism. The TOR-C1 form of TOR increases ribosomal synthesis, inhibits translational repression by phosphorylating *Thor*, and stimulates amino acid uptake via the amino acid transporter, Slimfast. There is extensive crosstalk and feedback among various branches of the insulin signaling pathway. Accumulation of amino acids activates TOR, thereby activating amino acid transport. dFOXO regulates the expression of *myc*, a target of TOR that stimulates ribosome synthesis (Teleman et al., 2008). dFOXO and TOR pathways also intersect via their opposing effects on the expression and activity of 4E-BP.

The alphabet-soup description above includes only a few components of the insulin/TOR signaling pathway, but it provides a framework for understanding how starvation affects signaling. During starvation in *Drosophila*, secretion of dILPs by the neurosecretory cells decreases. Food-seeking behavior increases, mediated by neural S6 kinase, a downstream target of insulin signaling. AKH secretion also stimulates activity (Lee and Park 2004; Isabel et al. 2005). Phosphatidylinositol-(3,4,5)-triphosphate levels decline, Akt becomes dephosphorylated, and dFOXO is recruited to the nucleus. *Thor* expression increases, and existing Thor protein becomes dephosphorylated and can inhibit elongation initiation factor eIF4B, thereby inhibiting protein synthesis. dFOXO and TOR inputs inhibit *myc* transcription, thereby inhibiting ribosome biogenesis. The overall result is a general reduction in energy-intensive biosynthetic activities. In addition TOR-mediated autophagy of fat cell contents commences, generating nutrients that can be used to support metabolism in the rest of the body (McPhee & Baehrecke, 2009; Scott, Schuldiner, & Neufeld, 2004).

This general pattern is likely to differ in a tissue-specific manner. It also can vary depending upon developmental stage. The pupa does not feed, yet needs to devote a significant fraction of metabolism to building adult tissues. Beginning in the wandering stage of the third instar, 20E signaling induces the larval fat body to express d*ILP6* (Slaidina, Delanoue, Groenke, Partridge, & Leopold, 2009) and activates lipid catabolism (S. Wang et al., 2010). Inhibition of d*ILP6* transcription in the fat body results in smaller adults, but these have high triglyceride levels and are more starvation resistant than control flies. Additional experiments revealed that d*ILP6* expression is regulated by dFOXO, providing a further example of the intersection between these pathways. In

another example of signaling crosstalk, recent work suggests that dFOXO regulates expression of *dDOR*, a coactivator of the ecdysone receptor (Francis, Zorzano, & Teleman, 2010).

Mammalian researchers will note that we have barely mentioned sugar homeostasis in our discussion of insulin signaling. To some extent this is due to the focus on the *Drosophila* larva, a very rapidly growing stage that requires high levels of amino acids to support biosynthesis. In fact, a common control treatment for "starvation" (lack of amino acids) is a diet containing sucrose to allow animals to continue to manufacture ATP.

In *Drosophila*, the primary signal for insulin secretion is the presence of amino acids, not carbohydrates. The primary site for sensing overall nutritional status is the fat body (Colombani et al., 2003). One or more factors secreted by the fat body stimulates dILP secretion by the NSCs when amino acids are abundant (Geminard et al., 2009). When amino acid levels are low or the Slimfast amino acid transporter is inactivated, dDILP secretion is reduced. Thus, the NSCs and fat body are in reciprocal communication with each other. The identity of the signal released by the fat body is unknown, but the fat body is known to produce numerous growth factors (Britton & Edgar, 1998; Kawamura, Shibata, Saget, Peel, & Bryant, 1999).

Under prolonged starvation, an additional energy source available to female flies is reabsorbed eggs (McCall, 2004; Wilson, 1985). Oogenesis is initiated from germline stem cells situated at the anterior tip of each ovariole, the germarium. An egg chamber or follicle forms, comprised of the oocyte and nurse cells enclosed in a layer of follicle cells (Wu, Tanwar, & Raftery, 2008). In well-fed laboratory strains of *D. melanogaster*, new

egg chambers are formed continuously over most of an adult female's lifespan. Reabsorption during starvation is initiated by apoptosis of the nurse cells (Terashima & Bownes, 2005a, , 2005b), and there is increased cell death in the germarium (Drummond-Barbosa & Spradling, 2001; Pritchett, Tanner, & McCall, 2009). One might predict that starvation-selected flies would contain fewer ovarioles than control flies, but this is not the case (Wayne et al., 2006). Reduced fecundity in these populations may instead be caused by lower activity of the germline stem cells or increased egg reabsorption, but this has not been investigated.

1.6 Genomics of Starvation Resistance

As the first multicellular eukaryote with a sequenced genome, *D. melanogaster* has been the subject of numerous genomic analyses, including several related to starvation stress. Harbison et al. (2004) identified nearly 400 genes associated with starvation resistance, many of them associated with cell fate determination. These could affect resource allocation during development, setting the conditions for survival later. This is consistent with selection experiments in which larval resource acquisition is a major determinant of adult starvation resistance (Chippindale et al., 1996). Analyses of quantitative trait loci (QTLs) have identified several genomic regions associated with differences in starvation resistance and energy storage (Harbison, Chang, Kamdar, & Mackay, 2005; Vieira et al., 2000; M. Wang, Harshman, & Nuzhdin, 2005).

Microarray experiments have shown that up to 25% of the transcriptome can be affected by starvation (Harbison et al., 2005). The first such transcriptome analysis was performed by Zinke et al. (2002). The focus of this study was sugar-related gene

expression, so larvae fed sugar were compared to starved and larvae fed sugar and a protein source. Several genes associated with lipid catabolism were upregulated specifically in starved larvae, whereas lipid synthetic genes were upregulated in larvae fed sugar. These results are consistent with the idea that starved larvae use the lipid they have to survive, while sugar-fed larvae use this resource to make ATP, with any excess going to lipid synthesis. Surprisingly, Harbison et al. (2005) found that genes for biosynthetic proteins tended to increase in expression in starved flies.

The studies above assayed whole-body gene transcription, but different tissues will respond differently to starvation (e.g. fat body and oenocytes). Immune function genes are downregulated in several tissues (Farhadian et al., 2012). In ovaries, changes in expression of multiple members of the insulin/TOR signaling are consistent with an inhibition of protein synthesis and cell growth (Terashima & Bownes, 2005b). Decreased expression of ovary-specific genes, such as yolk proteins, can also be detected in whole-animal experiments (Bauer et al., 2006). Starvation selection also affects gene expression. Sorensen et al. (2007) found that over 200 genes were constitutively down-regulated in starvation-selected lines, including many involved in transcription and glycolysis, suggesting that overall metabolism may be lower. Interestingly, the specific genes identified differed from those differentially expressed during starvation stress (Harbison et al., 2005). Thus, acute and evolutionary responses to starvation appear to rely on different mechanisms.

Genomic studies of starvation in natural populations of *Drosophila* have also been performed. In both North America and Australia, latitudinal clines in allele frequency of the insulin receptor have been observed in *D. melanogaster* (Paaby, Blacket, Hoffmann,

& Schmidt, 2010). In North America, this cline parallels a cline in starvation resistance (Schmidt et al., 2005; Schmidt & Paaby, 2008). No latitudinal clines were detected, however, for the InR substrate, Chico. This finding is consistent with genomic comparisons among *Drosophila* species, which show that evolution of downstream members of the insulin signaling pathway tends to be more constrained than that of upstream proteins (Alvarez-Ponce, Aguade, & Rozas, 2009; Alvarez-Ponce et al., 2012).

1.7 Summary

More is known about starvation responses in *Drosophila* than in any other insect, perhaps any other animal. The genetic resources available for *D. melanogaster* have made it a widely-used model to study regulation of energy storage and mobilization. For example, many aspects of TOR signaling were initially identified in *Drosophila*, then studied in mammalian systems (Martin & Hall, 2005). Genetic advantages notwithstanding, fruitflies are too small for convenient study of some aspects of starvation. For this reason, hemolymph transport of lipids is far better understood in larger insects such as *Manduca* (Arrese et al., 2001). Presumably *Drosophila* also convert TAGs to DAGs before releasing them into the hemolymph, but this has not been well studied. Life history differences among species will also affect how insects respond to starvation. Adult *Bombyx* moths do not feed, so starvation-induced reabsorption of eggs does not make sense and presumably does not occur. *Drosophila* is an excellent *model*, but comparative studies of insect starvation are still needed.

Comparative studies within the genus *Drosophila* should be very informative. *Drosophila* use a wide variety of host plants in nature, differing greatly in their spatial

and temporal availability, as well as nutritional content (Markow & O'Grady, 2008). Starvation resistance varies widely across the genus. Within species, local populations exhibit variation that in many cases suggests local adaptation to environmental conditions. At the time of this writing, genome sequences are available for 19 species of *Drosophila*, from many different nutritional habitats. A century of genetic research on *D. melanogaster*, intensive study of evolution in the genus *Drosophila*, and rapidly expanding genomic resources for *D. melanogaster* and its relatives provide many opportunities to deepen our understanding of starvation biology in insects and other animals.

CHAPTER 2

STARVATION RESISTANCE STRATEGIES

2.1 Starvation Resistance: *Drosophila* as a Laboratory Model

Three major strategies can be used to increase starvation resistance. *Drosophila* can store more energy (lipids, carbohydrates, protein), they can consume it at a slower rate (activity levels and metabolic rate), or they can tolerate loss of a greater fraction of their initial energy supply (lower energy stores required to remain alive) (Figure 1.2). These mechanisms are not mutually exclusive; in fact it is most likely a combination of these strategies that contributes to starvation resistance in a population. Laboratory natural selection has been used to investigate how these strategies might arise and the trade-offs associated with them.

Laboratory selection for stress resistance in outbred populations of *Drosophila* is a method used to study the evolution of starvation resistance and its correlative relationships in life history traits. Variation in selection results in outbred populations is common (Harshman & Hoffmann, 2000) and laboratory selection experiments are more complex than one might assume (Gibbs, 1999; Gibbs & Gefen, 2009). Previously conducted laboratory selection for starvation resistance has been plagued with inconsistencies in results (Gibbs & Reynolds, 2012; Harshman, Hoffmann et al., 1999). These differences may stem from variations in methods and population history, making it difficult to compare findings between studies. For example, starvation stress has been inflicted by several methods from nothing except wet cotton balls in their vials (Harshman & Schmid, 1998) to being fed only rotting lemons (Harshman, Hoffmann et

al., 1999) and either continuously or repeatedly for short intervals (Gomez et al., 2009). Which flies were stressed in the population also varied, with some studies selecting upon females (Harshman & Schmid, 1998), mixed sex groups (Chippindale et al., 1996), or only virgin males and females (Baldal et al., 2006). Varying which flies in the population were stressed affects gene flow in a population, having an ultimate effect on the rate of increase in starvation resistance. Despite a history of differences being found between the sexes in resistance to starvation (Chippindale et al., 1996; Harshman & Schmid, 1998) and females having been found the most starvation resistant (Chippindale et al., 1996), some studies only used males for some of the assays conducted (Harshman, Hoffmann et al., 1999). In addition to methods, differences in the history of populations likely affected the overall selection results, because the pool of possible alleles under selection has differed with previous selection for longevity (Chippindale et al., 1996; Djawdan et al., 1997) or from previous development as isofemale lines (Baldal et al., 2006).

An important limitation of previous studies was that they did not examine all potential starvation resistance mechanisms within a given set of populations. By characterizing all these strategies in one population set, I tried to clarify the current inconstancies between studies. In addition, I investigated how these strategies might trade off with other fitness traits.

2.2 Methods

2.2.1 Population Origin

The original outbred population of *Drosophila melanogaster* was collected from Terhune, New Jersey, USA in 1999. These flies were divided into multiple populations

for selection on desiccation resistance (Gibbs & Gefen, 2009). The two control population groups were used to create the starvation-selected and fed control populations used in this study. Each control group consisted of three replicate populations (A, B, and C). The first control group was a fed control on which no selection was imposed and is used for the same type of control in this study. The second control group had only been mildly selected for starvation resistance; populations were only subject to starvation for a short period of time in which less than 20% of the population died. This second control population group became the starvation-selected populations in this study. For more details on the populations' history see Gefen et. al. (2006).

2.2.2 Starvation Selection

Pre-adult stages were reared at densities of ~60 larvae in vials containing 10 ml of corn meal–sucrose–yeast medium. After 2 weeks, adult flies (approximately 4 days post-eclosion) were transferred to 5.5 L Plexiglas population cages containing two Petri dishes of food. A cloth sleeve covered one end and allowed access to the cage. Starvation-selected flies numbered around 8,000 and fed control flies around 2,000 flies. Once all flies for all populations were placed in their cages, the food media plates were removed and replaced with 1% agar Petri plates in the populations undergoing starvation selection. Fed control flies were maintained on food media plates. The plates (either agar or food) were changed every 2 days. When approximately 15-20% of the starvation-selected flies were left, food media plates were placed in the cages. After four days, yeast paste was added to fresh plates to stimulate egg production. Approximately 8,000 eggs were collected three days later for each population for the next starvation selected generation,

and ~2,000 eggs for each population for the next fed control generation. In order to prevent maternal and/or paternal effects in experiments, all flies used for experiments were one generation removed from this selection process. This was achieved by taking the progeny of flies subjected to starvation stress and using their subsequent offspring in experiments.

2.2.3 Starvation Resistance Assay

After 47 generations of starvation selection, ten males and ten females, virgin four-day old adults, from all six populations were placed individually in vials containing 10 ml of 1% agar at 25°C. Every four hours the number of dead flies was counted and every three days the surviving flies were placed in fresh agar vials.

2.2.4 Lipid, Glycogen, and Protein Assays

I measured total lipid, glycogen, and protein in 5 male and 5 female flies from all six populations after 47 generations of starvation selection. These samples were collected three times: newly eclosed adults, four-day old adults, and adults starved to death from the previously mentioned starvation resistance assay. In this chapter I report data from the four-day old adults and adults starved to death. Individual fly wet body masses were recorded for each individual before extractions using a Cahn micro balance (±0.001 mg). In the following chapter the data on four-day old adults and newly eclosed adults are compared in correlation with wet body mass.

2.2.4.1 Lipids

Flies were dried in a 50°C oven for 24 hours before dry weights were recorded. Each fly was placed in a glass vial with approximately 1 ml of diethyl ether for 24 hours. Flies were removed from the ether, placed in a 50°C oven for 10 minutes, and re-weighed. Lipid content was calculated by subtracting the weight after ether extraction from the original dry weight of the fly.

2.2.4.2 Glycogen

Individual flies were homogenized in 300 µl of lysis buffer (1% NP-40, 0.5% Deoxycholic acid, 0.1% Triton-X 100, 100 mM NaCl, 0.1 mM $CaCl_2$, and 2 mM $MgCl_2$ pH 7.6), and triplicates (15 µl) from each sample were loaded on 96-well microplates. 15 µl of *Rhizopus* amyloglucosidase (0.8 mg/ml; A-7255, Sigma-Aldrich Co., St Louis, MO, USA) were added to each well to catalyze the conversion of glycogen and trehalose into glucose (Parrou and Francois, 1997). The plates were sealed with parafilm and left overnight at 25°C. The following day, 180 µl of liquid glucose oxidase reagent (#G7521 Pointe Scientific Inc., Canton, MI, USA) were added to each sample and incubated at 37°C for 10 minutes. Absorbance at 500 nm was measured using a SpectraMax Plus384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Glycogen concentrations were determined using standards of known glycogen concentration.

2.2.4.3 Protein

The same lysates used in the glycogen assay were used for a bicinchoninic acid protein assay (BCA assay). 10 µl triplicates from each sample were loaded on a microplate, and 200 µl of BCA reagent was added. The plates were incubated overnight

at 25°C, and absorbance at 562 nm was measured the following day. Protein concentrations were determined using standards of bovine serum albumin (82516, Sigma-Aldrich Co.).

2.2.5 Fecundity

Fecundity was measured using Fly Condos (Doc Frugal Scientific, San Diego, CA, USA #59-110). After 45 generations of starvation selection, newly eclosed virgin females were placed in a collection tube. Eight females were assayed for each population. Each female was accompanied by two newly eclosed virgin male flies from the same population in their collection tube. Food plates were changed daily and contained grape juice agar topped with a small amount of yeast paste, a water and yeast mixture. The number of eggs laid by each individual female was recorded every 24 hours for 26 days.

2.2.6 Metabolic Rates

Metabolic rates were measured using flow-through respirometry after 53 generations of starvation selection. Groups of 10–20 virgin male or female four-day old adult flies were placed in 5 ml glass–aluminum chambers in a Sable Systems (Las Vegas, NV, USA) TR-2 respirometer. Flies were acclimated for one hour in chambers before a 15 minute period of measurement. Rates of carbon dioxide release were measured with a Li-Cor (Lincoln, NE, USA) LI-6262 infrared CO_2 sensor. There were 6 replicates for each population and sex. Following measurement, flies were killed and a lipid extraction (see previous methods) was conducted. Metabolic rates were then calculated by dividing the CO_2 released per hour by the total dry mass or lipid-free lean mass.

2.2.7 Activity Levels

AD-1 activity meters from Sable Systems were used to assess the relative activity of individual virgin male or female four-day old adult flies. Eight flies were assayed for each population after 50 generations of starvation selection. Individual flies were placed in 2 ml vials capped with a foam plug. The activity meters use a near infrared (900 nm) light source, which is reflected around the chamber to a detector. Activity was recorded for 15 minutes on empty vials; once a fly was placed in a chamber they were allowed 45 minutes of acclimation and then 15 minutes of recorded measurement. I quantified movement by comparing the signal when a fly was present to that of the same detector when empty (i.e. to machine noise). Whenever the signal for the fly was outside the 99% confidence interval of data for the empty chamber, we assumed the fly had moved.

2.2.8 Statistics

Statistica 7 (Statsoft Inc. Tulsa, OK, USA) was used to run statistical models. Replicate populations were treated as random effects and nested within the selection treatment. Sex was included as a variable in each model. Lipid, glycogen, protein, metabolic rates, and activity level assays were analyzed using an ANOVA. All ANOVAs included replicate populations and sex as factors in the model. When these factors were not significant they were combined and do not appear in graphs. Fecundity was analyzed with a repeated measures ANOVA. Starvation resistance survivorship was analyzed with a Cox-Mantel Test.

2.3 Results

2.3.1 Starvation Resistance Assay

Starvation-selected flies survived starvation longer than fed control flies by as much as seven days ($p<0.001$) (Figure 2.1).

2.3.2 Lipid, Glycogen, and Protein Contents

Lipid content was twice as high in starvation-selected four-day old adult flies compared to fed control flies ($p<0.001$). Although not surprising before starvation, it is interesting that after starvation nearly a quarter of the lipids remained in the starvation-selected flies ($p<0.01$) (Figure 2.2). There was a significant sex by selection interaction in the ANOVA model ($p<0.0001$) indicating a difference in lipid content between the sexes and selection treatment. A Tukey post-hoc test of flies starved to death showed that starvation-selected females had seven times more lipids than starvation-selected males or fed control flies ($p<0.001$) (Figure 2.3).

Glycogen content did not differ between starvation-selected flies and fed control flies at 4 days of age or after being starved to death ($p>0.05$) (Figure 2.4). A few interactions in the ANOVA model were significant, however upon examination with a Tukey post-hoc analysis no significant differences were found.

Protein content did not vary before or after starvation, indicating that protein is not used for energy to survive starvation ($p>0.05$) (Figure 2.5). A few interactions in the ANOVA model were significant, however upon examination with a Tukey post-hoc analysis no significant differences were found.

Figure 2.1

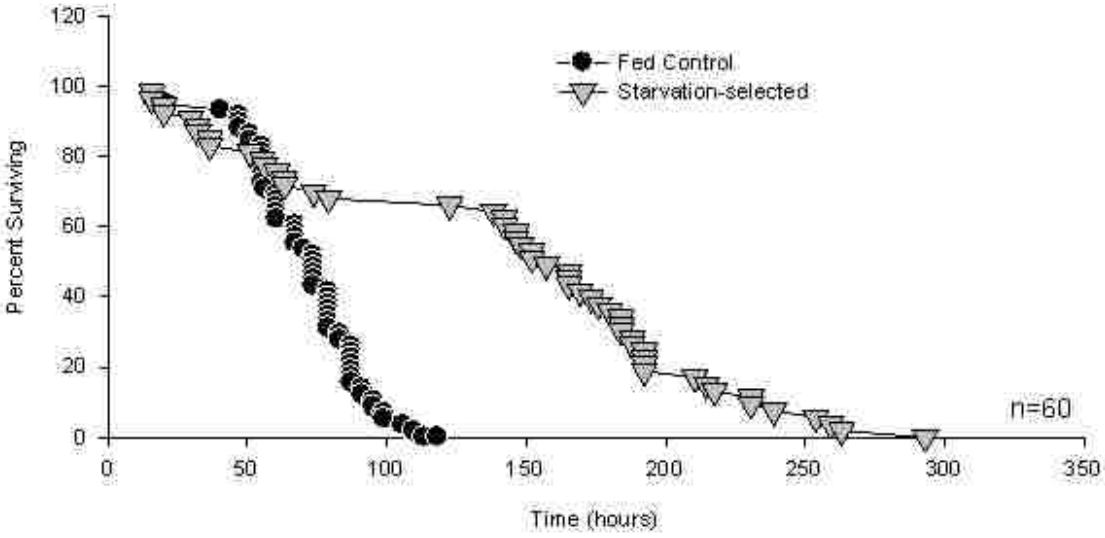


Figure 2.1 Starvation resistance of flies: 4 day old virgin adult flies starved to death in vials with only agar. Starvation-selected flies survive starvation for a longer period than the fed control flies ($p<0.001$).

Figure 2.2

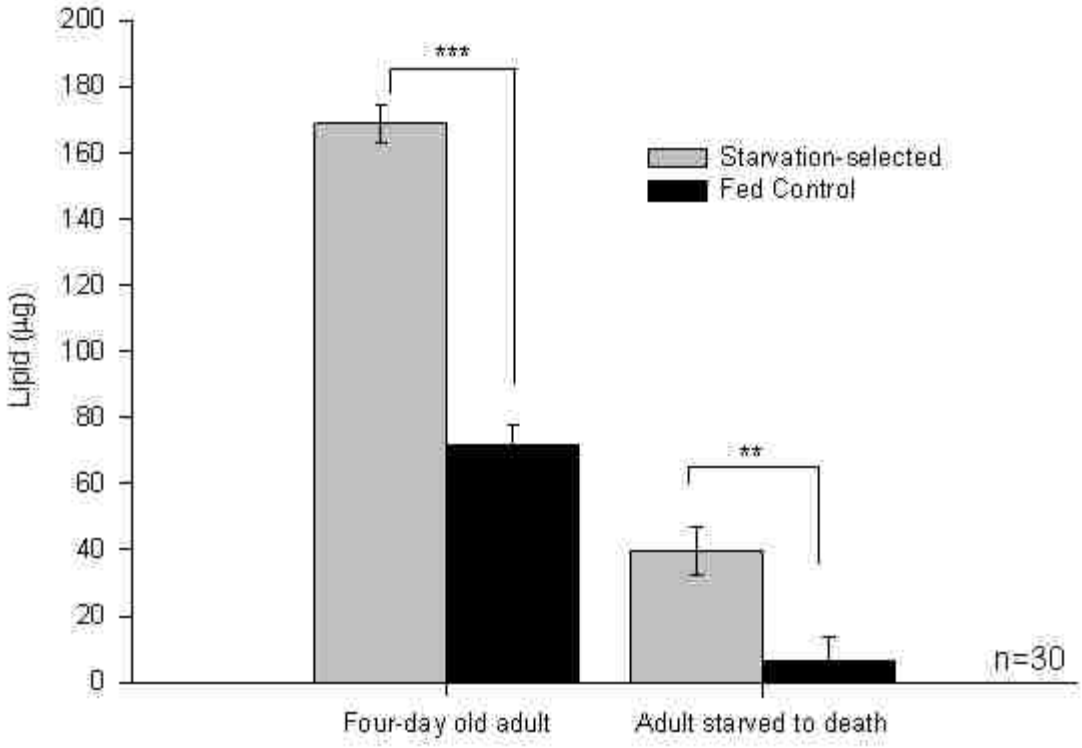


Figure 2.2 Lipid content of flies: Least square means of lipid content of 4 day old adult flies and adults starved to death. Error bars are standard errors from ANOVAs. Replicate populations and sexes were combined, because they were not significantly different in the ANOVA model.

Figure 2.3

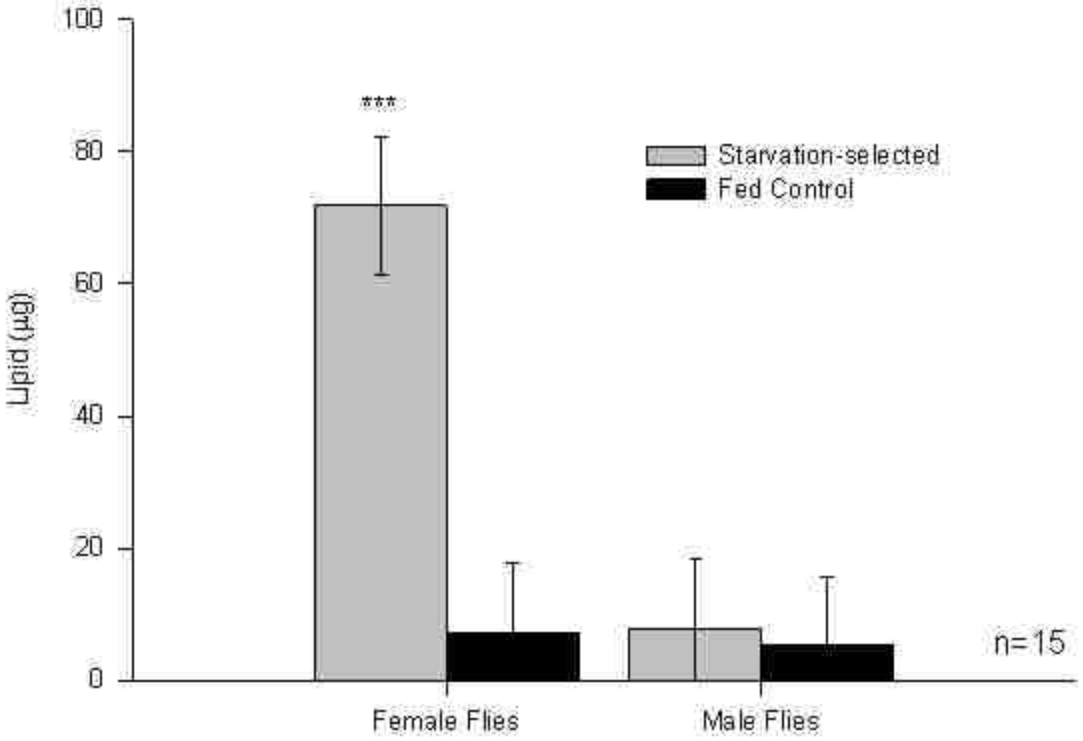


Figure 2.3 Lipid content of flies starved to death: Least square means of lipid contents of adults starved to death. Starvation-selected female flies contain more lipid after being starved to death compared to all other groups. Error bars are standard errors from ANOVAs. Replicate populations were combined, because they were not significantly different in the ANOVA model.

Figure 2.4

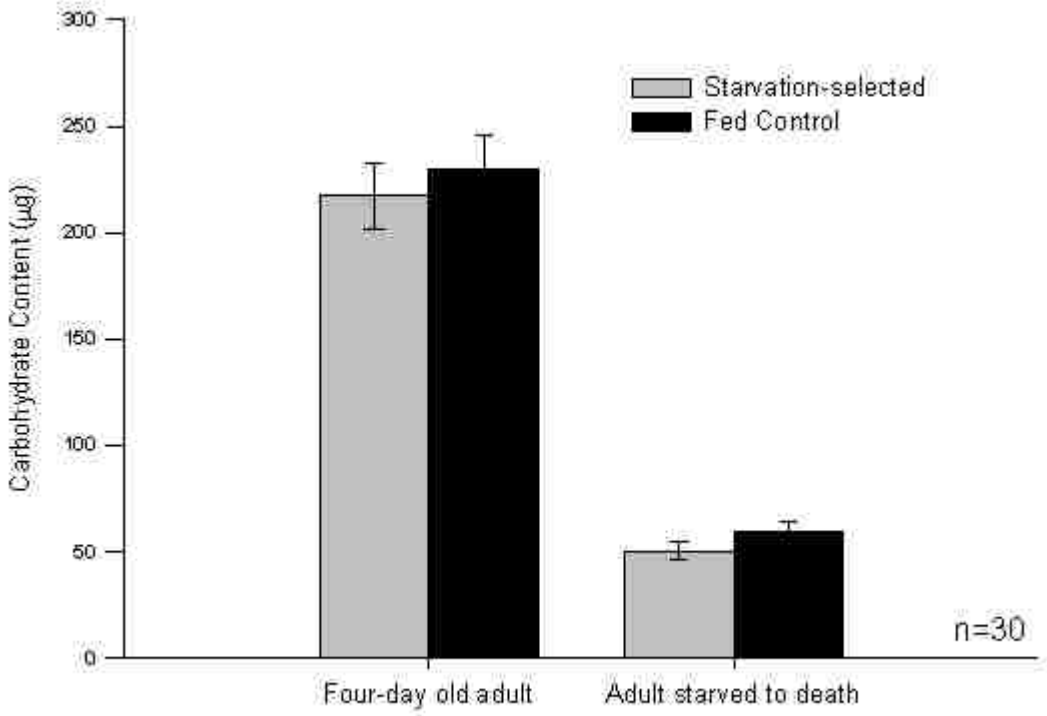


Figure 2.4 Glycogen content of flies: Least square means of glycogen contents of 4 day old adult flies and adults starved to death. Error bars are standard errors from ANOVAs. Replicate populations and sexes were combined, because they were not significantly different in the ANOVA model.

Figure 2.5

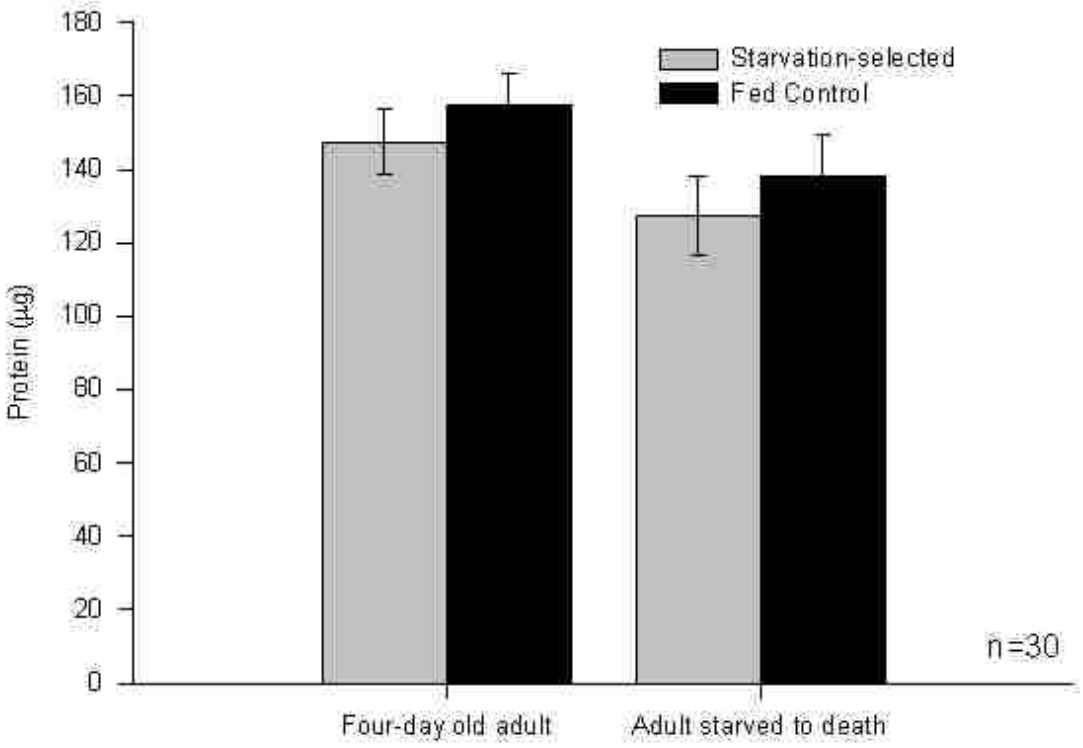


Figure 2.5 Protein content of flies: Least square means of protein contents of 4 day old adult flies and adults starved to death. Error bars are standard errors from ANOVAs. Replicate populations and sexes were combined, because they were not significantly different in the ANOVA model.

2.3.3 Fecundity

Starvation-selected flies showed decreased fecundity compared to fed control flies ($p<0.05$) (Figure 2.6). Parallel fluctuations in daily egg lay were likely due to parallel variation in the grape agar media on which females laid their eggs.

2.3.4 Metabolic Rates

Mass specific metabolic rates (µl CO_2-hr^{-1}-µg $mass^{-1}$) were calculated using both total mass and lean mass (no lipids) for individual flies. Starvation-selected flies had lower metabolic rates whether lipids were included as part of the total mass or not (total dry mass $p<0.001$, lean dry mass $p<0.001$) (Figure 2.7). No significant differences between the sexes or replicate populations were seen and the values were combined in Figure 2.7.

2.3.5 Activity Levels

Starvation-selected flies tended to be less active, but this difference was not statistically significant ($p=0.056$) (Figure 2.8).

2.4 Discussion

Starvation-selected *Drosophila* can increase their starvation resistance by three mechanisms. Flies can store more energy (lipids, carbohydrates, protein), they can consume it at a slower rate (lower activity levels and metabolic rates), or they can tolerate loss of a greater fraction of their initial energy supply (lower energy stores required to remain alive) (Figure 1.1). These mechanisms are not mutually exclusive; in fact it is

Figure 2.6

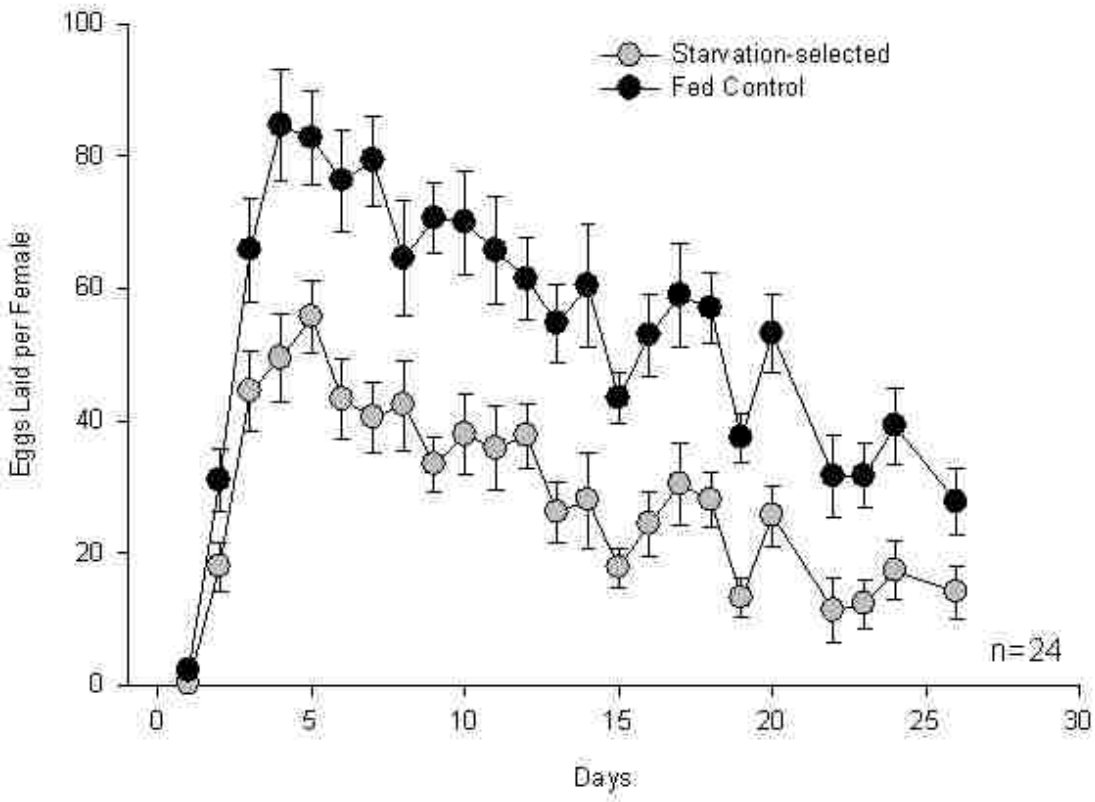


Figure 2.6 Fecundity of flies: Fecundity of newly eclosed females, females from starvation-selected flies consistently have lower fecundity. Least square means with standard error bars. Replicate populations were combined, because they were not significantly different in the ANOVA model.

Figure 2.7

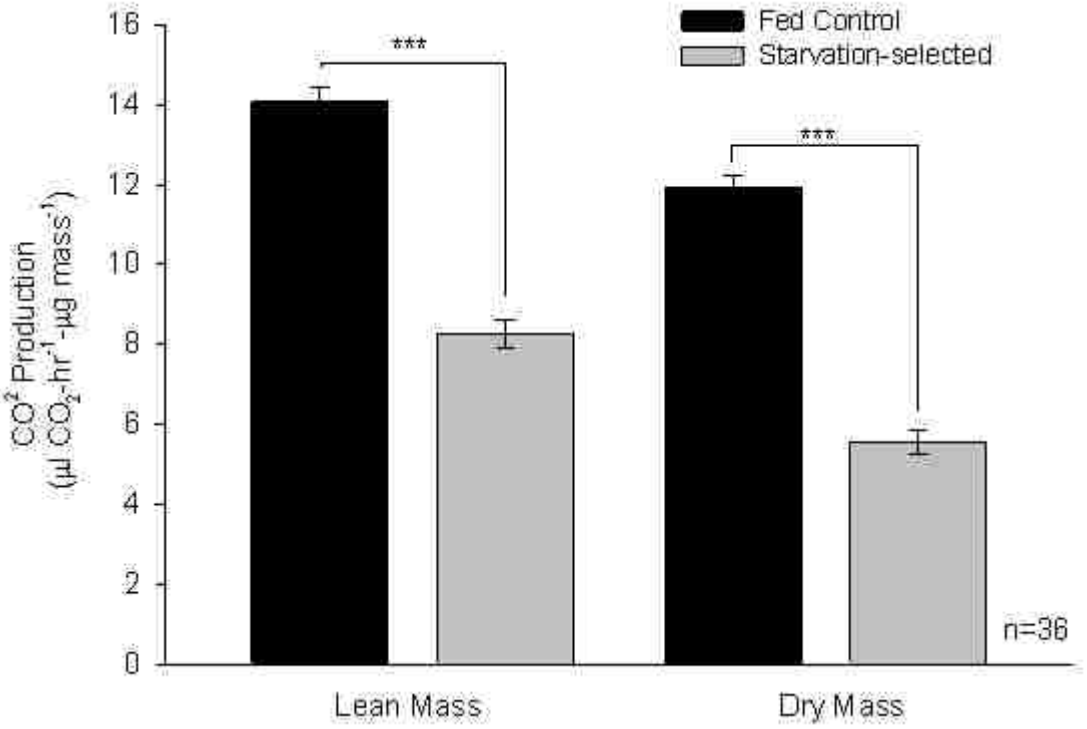


Figure 2.7 Metabolic rates of flies: Least squared means of metabolic rates calculated with total lean mass or dry mass. Starvation-selected flies have significantly lower metabolic rate when calculated both ways ($p<0.001$). Replicate populations and sexes were combined, because they were not significantly different in the ANOVA model.

Figure 2.8

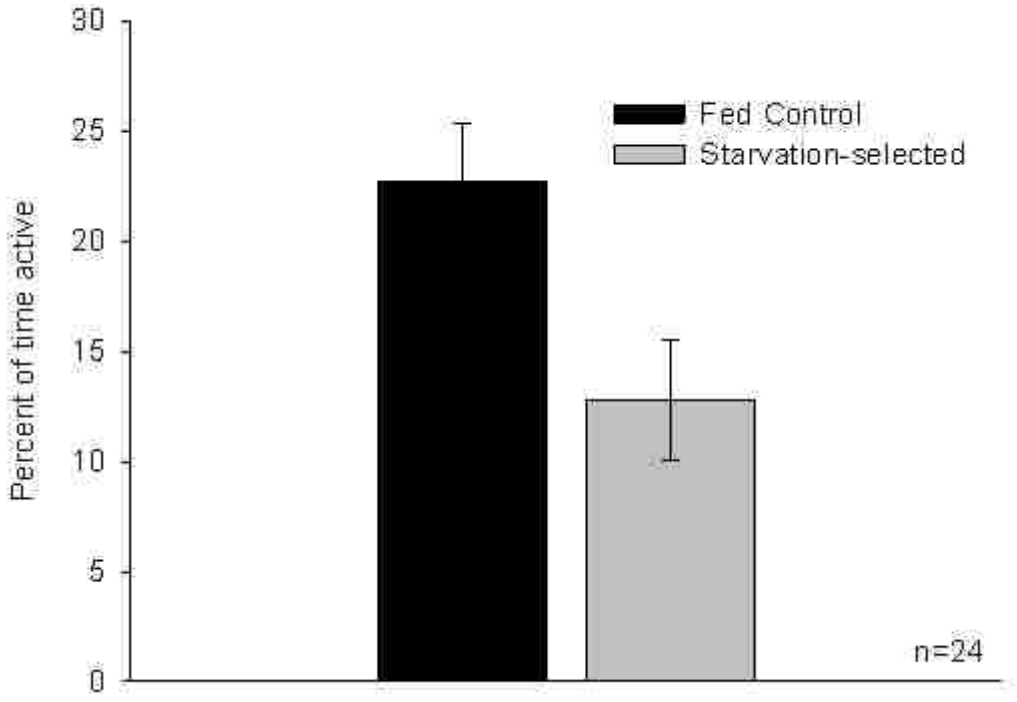


Figure 2.8 Activity levels of flies: Starvation-selected flies appear to be less active then their fed control fly counter parts, however the difference is not significant (p=0.056). Replicate populations and sexes were combined, because they were not significantly different in the ANOVA model.

most likely a combination of these strategies that has increased starvation resistance in our starvation-selected flies.

My data show that two mechanisms, increased lipid stores (Figure 2.2) and decreased metabolic rates (Figure 2.7) are the major traits responsible for the increase in starvation resistance in the selected flies. Starvation-selected flies have higher lipid content, consistent with previous studies (Baldal et al., 2006; Chippindale et al., 1996; Griffiths et al., 2005; Harshman, Hoffmann et al., 1999). Interestingly, the starvation-selected female flies have significantly more lipids after starvation than any other group of flies, including fed control females (Figure 2.3). It is difficult to imagine why such a resource was not used during starvation. One possibility is the females died from the depletion of a micronutrient. Another would be that the lipids stored were unavailable for use to resist starvation; perhaps the lipids may have been stored in the ovaries. When larval fat-cell death is blocked, preventing this energy from being deposited in ovaries, starvation resistance is increased in females (Aguila et al., 2007). This explanation, however, does not account for why fed control females do not have more lipids after being starved to death. One possible reason for this is fed control females lay their eggs early in life, thus before starvation stress, and may continue to lay eggs under starvation conditions. Starvation-selected females however, may retain eggs during starvation until food is available.

Glycogen was also consumed during starvation, but the starvation-selected and fed control flies did not differ in the amount of glycogen they contained, nor did they differ in their usage of glycogen (Figure 2.4). This suggests that glycogen content is not a

trait selected upon; if it were, the starvation-selected flies would have significantly more than the fed controls or have used more of it than fed controls after starvation.

Metabolic rates have been measured several times in starvation-resistant fly populations, however results have varied (Baldal et al., 2006; Djawdan et al., 1997; Harshman, Hoffmann et al., 1999; Harshman & Schmid, 1998). There has been debate about whether lipid mass (typically much larger in starvation-selected flies and considered metabolically inactive) should be included in metabolic rate calculation (Djawdan et al., 1997). By including extra mass that is metabolically inactive in the calculation, the metabolic rates can be artificially lowered. I used both total dry mass and lean dry mass to calculate metabolic rates and found that in either case metabolic rates were significantly lower (Figure 2.7). In addition to a lower metabolic rate, the starvation-selected flies also have a strong tendency towards a lower activity level under the conditions of our assay (Figure 2.8).

The combination of these characteristics in the starvation-selected flies fits nicely into using a combination of increased energy storage and lower rate of energy consumption as an overall starvation resistance strategy (Figure 1.1, A&B). I did not, however, find evidence for a decrease in the amount of energy needed to prevent death from starvation, as seen by the equal amounts of lipids, carbohydrates, and proteins upon death from starvation in both starvation-selected and fed control flies (Figure 1.1, C). In fact, starvation-selected females had more lipids after death from starvation than any other group of flies as discussed earlier (Figure 2.3).

These changes in energy storage and consumption have life history trade-offs. Correlated with the increase in lipid storage and changes in metabolism, we see a

negative impact on fecundity (Figure 2.6) and an increase in development time (explained in greater detail in chapter 4). Fecundity has been reported before as a trade-off with starvation resistance (Kolss et al., 2009; Wayne et al., 2006), but changes in development time are not always present (Griffiths et al., 2005). The connection of increased starvation resistance correlated with a decrease in reproduction is likely due to changes in resource allocation within the fly. Aguila et al. (2007) increased starvation resistance by blocking larval fat cell death, with a consequence decrease in fecundity (Aguila et al., 2007). A decrease in the expression of genes associated with oogenesis has also been seen under starvation stress (Harbison et al., 2005). The decrease in fecundity and increase in starvation resistance presents a classic example of a trade-off of resource allocation within the individual between reproduction and survival (Stearns, 1992). Surviving starvation to live long enough to reproduce successfully becomes very important in the starvation-selected populations, so even though they have large energy reserves, only a small fraction of their energy reserves are allocated to reproduction, creating a negative correlation between fecundity and surviving starvation (van Noordwijk & de Jong, 1986).

Starvation selection affects many life history and physiological traits. The fly populations have achieved increased starvation resistance; where lipids are preferentially stored for energy and decreased metabolic rates conserve energy. However, these changes have a marked trade-off in decreased fecundity. This detailed characterization of life history traits to a single set of starvation-selected *Drosophila* helps to eliminate some of the difficulties in identifying trade-offs, mechanisms, and life history trait interactions

that would otherwise go unnoted in comparisons between multiple different studies of starvation resistant *Drosophila* populations.

CHAPTER 3

LIFE HISTORY INTERACTIONS

3.1 Importance of Interactions in Life History Traits

In the evolution of life history traits, certain parallel relationships in life history traits are common, such as proportional growth, as well as antagonistic/trade-off interactions, such as number and quality of off spring. Deviations and uncoupling of commonly seen interactions are of interest to evolutionary biologists, because they give insight into the conditions when fitness is optimized by a different interaction pattern. This information can then be used to adapt or support models for predicting when a shift in an interaction occurs. By studying interactions between life history traits, a greater understanding develops of how a life history phenotype contributes to reproduction and survival.

One of the most important factors affecting life history traits is body size respirometry (Calder, 1996). Larger animals tend to have greater energy reserves that can be used for survival or reproduction. They are more mobile and have larger home ranges, and therefore access to greater amounts and types of resources. Larger individuals may have greater reproductive success by outcompeting other members of their species for mates, or by sexual selection for larger mates. In insects, larger females generally have higher fecundity. Trade-offs against the benefits of large body size include slower development to reproductive age and the need to acquire more resources to maintain metabolism.

In the previous chapter, I determined the major starvation resistance strategies used by the starvation-selected flies. Starvation-selected flies had a greater total wet mass than their fed control counterparts. Lipid and glycogen were consumed during starvation, and larger amounts of lipids were found in the starvation-selected flies. Thus, it is possible that starvation selection has resulted in populations of flies that are simply larger than their controls. Larger body size is often correlated with higher fecundity, yet starvation-selected females had lower fecundity. This could be an example of a trade-off in resource allocation between reproduction and survival in females (van Noordwijk & de Jong, 1986).

In this chapter I discuss how the starvation-selected flies vary from this predicted correlation with relation to fecundity and other life history traits and the life history implications for starvation resistance. I used an analysis of covariance (ANCOVA) to explore the relationships between body size and energy storage, fecundity and age. ANCOVAs are typically used to determine whether a continuous covariable, such as body size, affects the dependent variable of interest. This approach can be used to statistically remove the effects of the covariate, so that underlying interactions between other variables can be identified. I used this analysis to outline an energy resource trade-off model in which evolution of starvation resistance proceeds by different physiological mechanisms as selection proceeds over time.

3.2 Methods

3.2.1 Lipid, Glycogen, Protein, and Body mass Assays

Total lipid, glycogen, and protein in 5 male and 5 female flies from each population replicate were measured after 47 generations of starvation selection. Adults were assayed when newly-eclosed and at 4 days of age. In chapter 2 data from the four-day old adults were reported, but in this chapter the interactions that occur with adult age and sex are included in the results. Individual fly wet masses were recorded for each individual fly before energy extraction using a Cahn micro balance (±0.001 mg). See chapter 2 methods for more details on the individual energy content assays.

3.2.2 Statistics

Statistica 7 (Statsoft Inc. Tulsa, OK, USA) was used to run all ANOVA and ANCOVA models. The dependent variables of interest were lipid, glycogen and protein content, which were analyzed in separate ANCOVA models. Sex, adult age, and selection treatment were treated as fixed main effects, while replicate population (nested within selection treatment) was treated as a random effect. Body size (wet mass) was included in the models as a covariate. Replicate population had no statistically significant effects in any of these models, so data from replicates were pooled in subsequent analyses.

In each ANCOVA model, body size and one or more main effects showed statistically significant interactions. These relationships indicate that size affected experimental groups in different ways (i.e. slopes relating body size and energy storage were non-uniform). This violates the assumptions of ANCOVA, so further ANOVA models were analyzed and their tables are found in Appendix A. In these cases, ANOVAs were performed using wet mass, lipid, glycogen, or protein as dependent variables. Sex, adult age, and selection treatment were treated as fixed independent variables, with

population treated as a random variable, nested within selection treatment. Least square means calculated from these ANOVAs were graphed against each other and used to explore the relationships between body size and energy storage. Tukey post-hoc tests were used to compare significant effects within the ANOVA models.

3.3 Results & Discussion

Table 3.1 contains the ANCOVA table that resulted from comparing lipid content and body mass while testing for significant interactions with independent variables. Highlighted in the table are every significant interaction between an independent variable (sex, age, and selection treatment) and the covariate (body mass). Every interaction was significant except in the case of population replicates. If an interaction is significant, it represents a change in the relationship between the dependent variable (in the case of Table 3.1 lipids) and covariable (body mass) based on the independent variable in the interaction. For example, there is a significant interaction between selection treatment and body mass in Table 3.1. This signifies that the relationship between body mass and lipid content (either a positive, negative, or neutral correlation) is significantly different between the starvation-selected and fed control flies. Inspection of the raw data indicated that lipid content increased more rapidly with wet mass (i.e. the slope of the relationship was greater) in the starvation-selected populations than in their controls.

3.3.1 Selection Treatment and Body Mass Interaction

The starvation-selected flies universally have greater total wet body mass (roughly 25%) than their fed control counterparts ($p<0.001$) (Figure 3.1-3), however

Table 3.1

	degrees of freedom	F	p
Intercept	1	11.13	0.002952
Selection	1	18.88	0.002225
Population (Selection)	4	2.24	0.069670
Sex	1	14.20	0.000273
Age	1	52.15	0.000000
Body Mass	1	55.41	0.000000
Age*Body Mass	1	65.94	0.000000
Sex*Body Mass	1	9.97	0.002083
Population (Selection)*Body Mass	4	2.39	0.055315
Selection*Body Mass	1	6.18	0.035477
Error	104		

Table 3.1 ANCOVA covariate interactions: ANCOVA result table for the dependent variable total lipid content. The ANCOVA model is used to find which interactions with the covariable total wet body mass are significant (these interactions are highlighted). The independent variables (age, sex, and selection) have significant interactions with the covariate (body mass). See methods 3.2.2 statistics for a more detailed explanation.

Figure 3.1

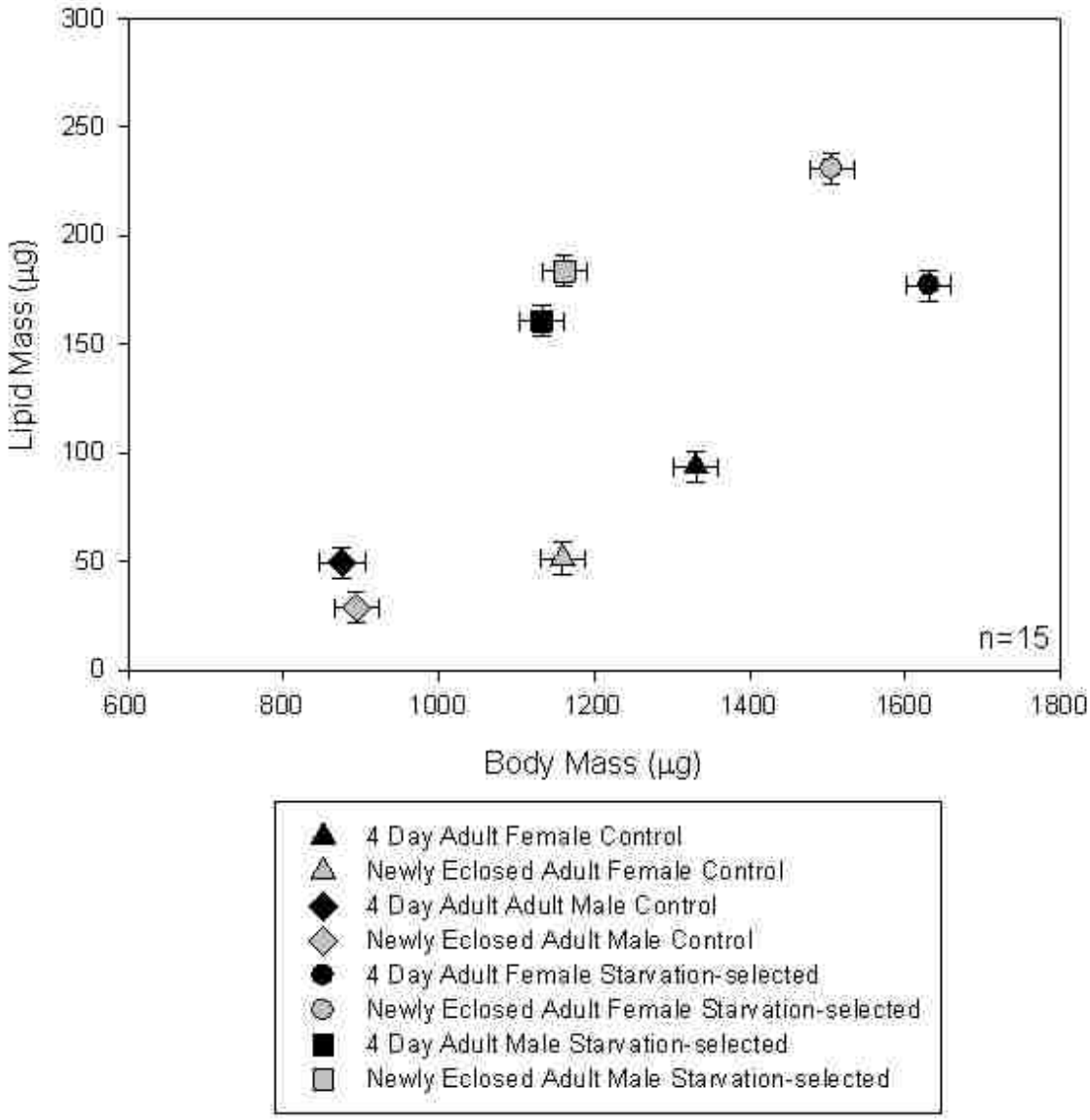


Figure 3.1 Body Mass vs Lipid Content: Least square means are from separate ANOVAs on either body mass or lipid content. Replicate populations were combined, because there was no significant effect. Error bars are the standard error calculated from the separate ANOVAs.

Figure 3.2

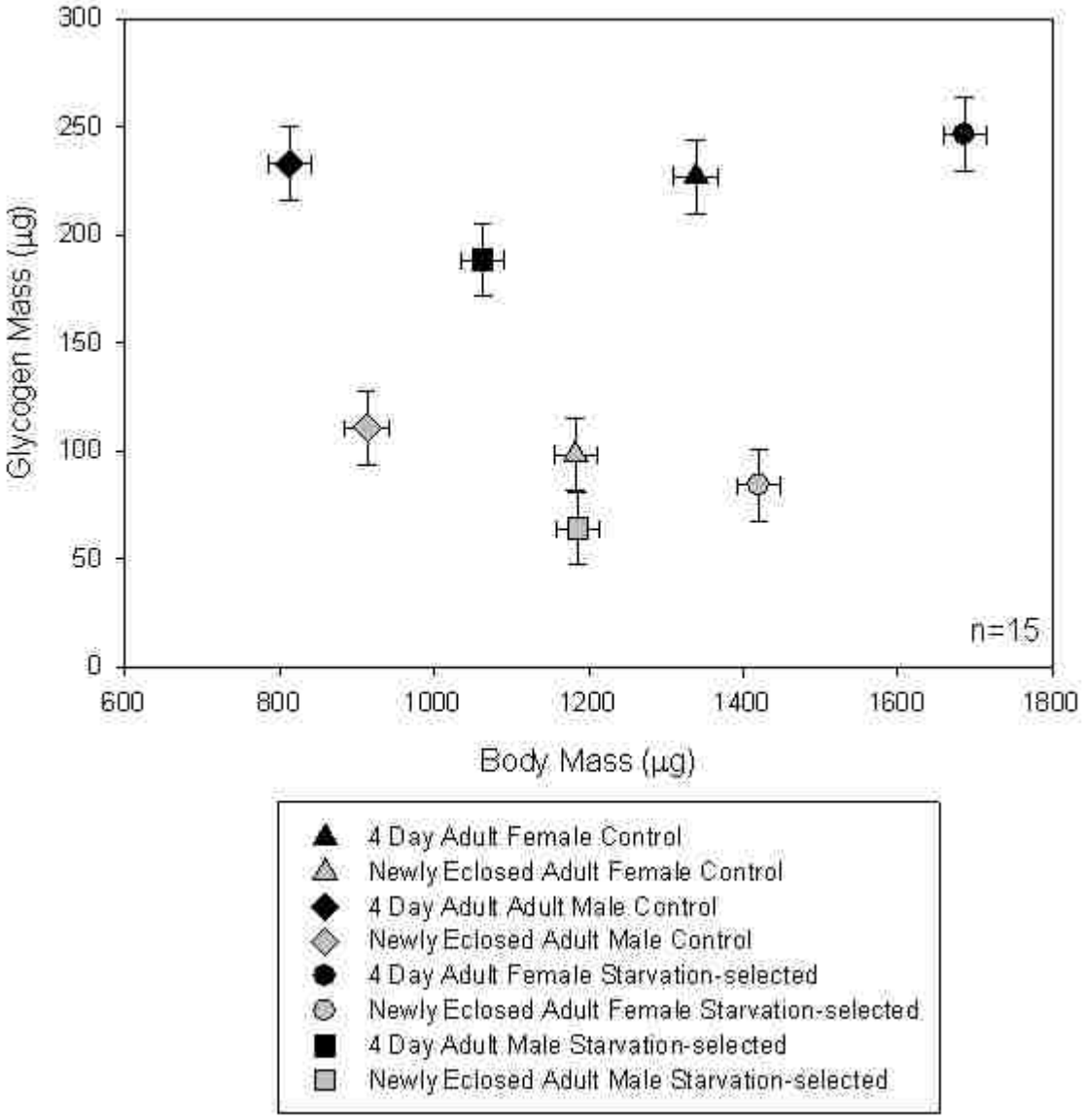


Figure 3.2 Body Mass vs Glycogen Content: Least square means are from separate ANOVA on either body mass or glycogen content. Replicate populations were combined, because there was no significant effect. Error bars are the standard error calculated from the separate ANOVAs.

Figure 3.3

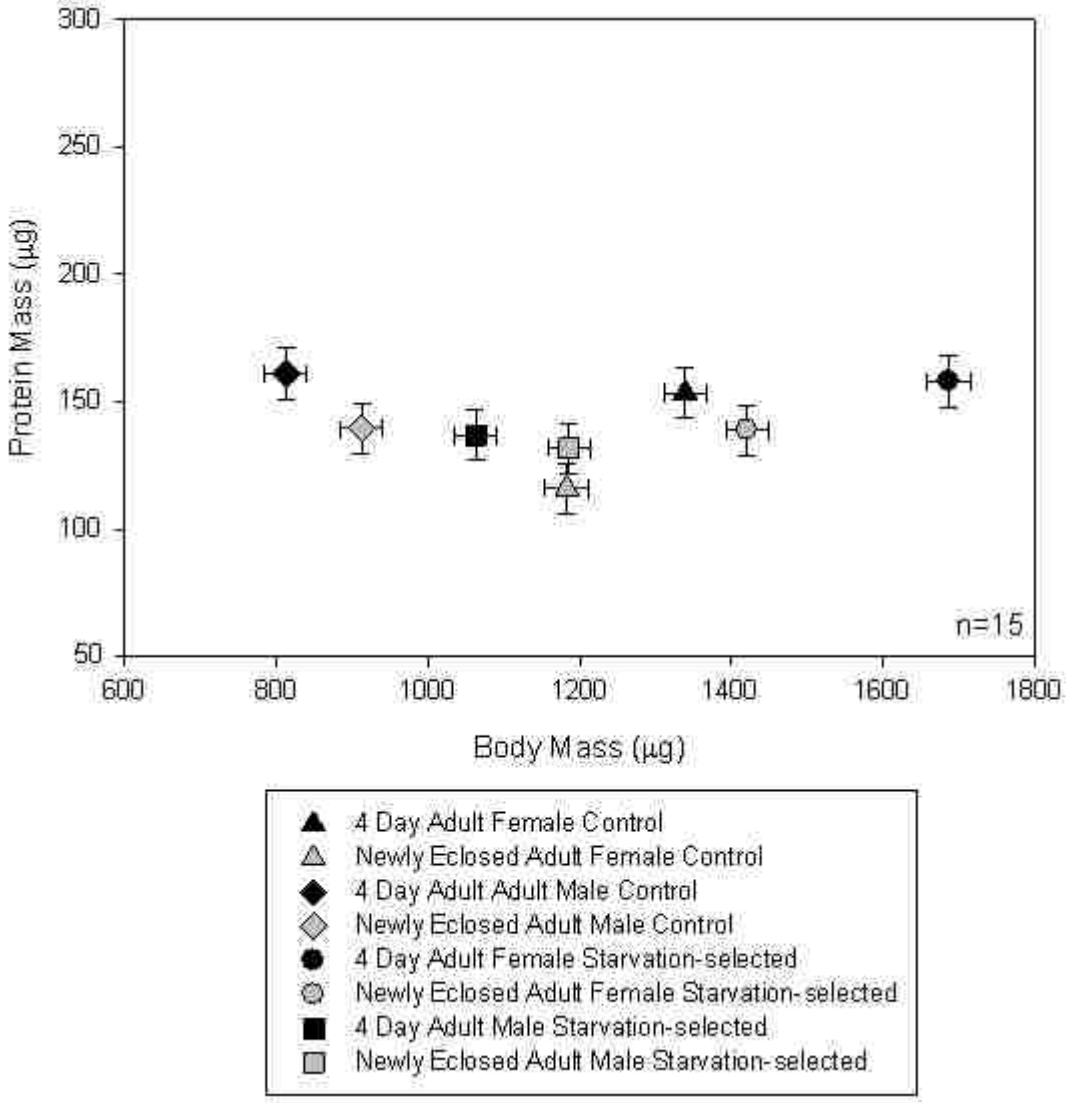


Figure 3.3 Body Mass vs Protein Content: Least square means are from separate ANOVA on either body mass or protein content. Replicate populations were combined, because there was no significant effect. Error bars are the standard error calculated from the separate ANOVAs.

despite their greater body mass they do not have a higher glycogen or protein content (Figure 3.2-3); instead they have a significantly higher lipid content (roughly 200%) (Figure 3.1). This indicates that the difference in body mass between selection treatments is not uniformly represented within the energy content measures. This variation in the allometry of resources and body mass between the selection treatments implies that lipids are the most important of these resources for surviving starvation.

Although an increase in lipids stores has been seen in other starvation-selected lines (Baldal et al., 2006; Chippindale et al., 1996; Griffiths et al., 2005; Harshman, Hoffmann et al., 1999), the relationship here with body mass has not been measured/seen before. The 200% increase in lipid content and the lack of increase in protein or glycogen in proportion to the increased body mass in the starvation-selected flies, implies a trade-off between types of energy stored. This could provide a tipping point in the continued starvation selection of these flies. As mentioned in chapter 2 the starvation-selected flies use lipid storage as a starvation resistance strategy, however if the maximum portion for storing lipids is limited by a minimum portion of glycogen and protein, the starvation-selected flies could shift their energy storage starvation resistant strategy and rely more heavily on the second starvation resistant strategy of using energy reserves more slowly (Figure 1.2 A&B). Evolutionarily, once the starvation-selected flies have reached their maximum lipid storage, a plateau would be predicted in the length of time the starvation-selected flies can resist starvation each generation and a subsequent increase in the selective pressure would result for phenotypes that slowed the expenditure of the stored resources.

3.3.2 Adult Age and Body Mass Interaction

In *Drosophila* adult morphometric characters indicating size are fixed upon maturity, however total body mass can change with adult age. The flies from which lipids were extracted show an overall significant increase in their total wet body mass from newly eclosed to 4 days of age ($p<0.05$) (Figure 3.1). This was not true though in the overall average of the flies from which glycogen and protein were extracted (Figure 3.2-3). Yet, whether or not this overall body mass average change with adult age was significant, there was a significant interaction between age and sex on their body masses ($p<0.001$), which will be discussed in the next section on interactions between the sexes.

With only some indication that body mass changes with adult age in all flies (only significant in flies from which lipid was extracted), it is interesting which type of energy content changes with age. In the case of lipids (for which there was a significant increase in body mass with age), there was no significant increase in lipid content with age (Figure 3.1). This indicates that no significant amount of lipid is gained or lost between eclosion and 4 days on average in all flies from which lipids were extracted, suggesting that lipids are mostly accumulated during a different life stage. Considering that energy is consumed, not accumulated, during the pupal stage, lipids must be mostly accumulated during larval development. This is of particular interest since in the previous section it was shown that the starvation-selected flies have overwhelmingly more lipids than the fed control flies (Figure 3.1). A possible physiological mechanism for larval lipid accumulation is presented in Chapter 4.

In contrast to the interaction between total body mass and lipids, glycogen and protein content change significantly with age ($p<0.001$), yet no significant change in

body mass with age was seen (Figure 3.2-3). Glycogen content increased roughly 60% with age in all flies measured ($p<0.001$) (Figure 3.2). Since this increase was seen in both starvation-selected and fed control flies and the level of increase did not differ, it is not likely that glycogen contributes significantly to the starvation-selected flies' increased starvation resistance. It is interesting that this resource is mostly accumulated during adulthood rather than larval stages, as seen with lipids. Particularly interesting was that overall body mass did not increase, although glycogen content significantly increased, suggesting that there was proportional loss of mass elsewhere to make up for the gain in glycogen, or the sex of the fly had a significant counteracting effect (discussed in the follow section). Protein content also increased with adult age ($p<0.05$) and upon further investigation, the fed control flies increased their protein content with age ($p<0.05$), but the starvation-selected flies did not change their protein content ($p>0.05$) (Figure 3.3). As noted in the previous section (Section 3.3.1), there is a possible trade-off between types of energy stored in flies. It is possible that the protein increases with age in fed controls, because none of the energy resource types have reached a maximum. However in the starvation-selected flies, where massive amounts of lipids are stored, a maximum may have already been reached with lipid and glycogen stores, which are essential for resisting starvation, and therefore protein content must remain low.

3.3.3 Sex and Body Mass Interaction

Drosophila females tend to have a larger body size than their male counterparts, and so it comes as no surprise that the total wet body mass of flies varied significantly with regard to sex ($p<0.001$) (Figure 3.1-3). In all cases females had roughly 40% more

body mass than males. Body mass not only is significantly different between the sexes, but also varies with adult age ($p<0.01$) (Figure 3.1-3). This indicates that body mass changes with adult age and the direction of this change is significantly different depending on the sex of the flies. In the previous section (Section 3.3.2) body mass overall did not change with age in flies from which glycogen and protein were extracted, however if examined with regard to the sex of these flies, a significant increase in female body mass with age is seen ($p<0.001$) (Figure 3.2-3) which is also seen in female flies from which lipids were extracted ($p<0.001$) (Figure 3.3). In males the relationship between body mass and age is not as straight forward. Male flies, from which glycogen and protein were extracted, significantly decreased their body mass with age ($p<0.001$) (this opposite trend compared to their female counterparts accounts for there being no overall significant change in wet body mass with age), however males from which lipids were extracted show no change in body mass with age ($p>0.05$). It is difficult to understand why body mass would decrease or remain unchanged in male flies when a marked increase in body mass is seen in female flies, however this increase in female body mass could easily contribute to increased female starvation resistance. Female starvation resistance has before been found to be the driving force in starvation-selected *Drosophila* populations (Chippindale et al., 1996).

It is possible females may have greater capacity for energy storage than males, because of their greater initial body mass and its increase with age. Female flies did not have significantly more glycogen or protein compared to males ($p>0.05$), despite their greater body mass (Figure 3.2-3). However, female flies did have significantly more lipid then males ($p<0.001$) (Figure 3.1). Since lipids have already been shown in the previous

chapter and Section 3.3.1 to be particularly important to resisting starvation, it suggests that females should be more starvation resistant than males. This relationship has previously been found in other populations (Chippindale et al., 1996), but not in the populations I studied (see chapter 2). One potential explanation is females do not use all the lipids they contain to resist starvation. In chapter 2 starvation-selected females were found to have lipids leftover after starvation (Figure 2.3).

Although overall lipid content does not vary with age, there was a significant sex-by-age interactive effect on lipid content $p<0.001$) (Figure 3.1). Starvation-selected flies lost lipid content as they aged ($p<0.001$), while lipid content increased with age in fed control flies ($p<0.001$). This significant opposite interaction is driven by the females in both the selection treatments and is best summarized by saying the starvation-selected females lose lipids with adult age and fed control females gain lipids with adult age ($p<0.001$) (Figure 3.1). The loss of lipid content in the starvation-selected females while gaining body mass is surprising. A potential explanation is a trade-off between energy stores, where glycogen must be acquired by all flies after eclosion. The starvation-selected female flies may not be able to acquire additional net mass, and so may lose lipid content in order to gain glycogen. Fed control flies, which have not reached a maximum in any energy content category, can continue to accumulate energy stores with impunity during adulthood. This result suggests that the starvation-selected flies, particularly the females, may be reaching a maximum in the amount of lipid content that can be stored to resistant starvation. As this strategy of lipid accumulation (Figure 1.2 A) is exhausted, selective pressure will increase on other starvation resistant strategies (Figure 1.2 B&C) potentially changing the course of evolution in these flies.

3.3.4 Summary & Evolutionary Implications

The original intention in collecting data on energy content was to determine which type of energy was important to starvation resistance and which selection treatment had the most of each energy type. Information on sex, age, and body mass data was collected and used as control variables. These data are discussed in this general manner in chapter 2 and no further experiments were designed to bring conclusive evidence to any underlying significant interactions, however I would have been remiss to ignore all the significant interactions in an ANCOVA and what further information could be extracted from these data.

The development period during which lipid and glycogen are mostly accumulated is an important discovery for understanding how starvation resistance is achieved in the starvation-selected flies. Lipids are shown here to be mostly accumulated during larval development in the starvation-selected flies, while glycogen was accumulated during adulthood (Figure 3.1-2). The difference in how much and when lipids are accumulated between the selection treatments, emphasizes the importance of lipids in contributing to the increased starvation resistance of the starvation-selected flies; glycogen by contrast does not vary between selection treatments. In the following chapter 4, I present a possible physiological mechanism for the increased lipid accumulation in the starvation-selected flies.

Analysis of interactions also brought to light the stark contrast between the sexes. Females were found to universally have greater body mass, which increased with adult age, and fed control females continually accumulated lipids, glycogen, and protein with

adult age (Figure 3.1-3). Since energy accumulation is a major strategy for resisting starvation, it implies females possibly have greater starvation-resistance (Figure 2.1 A). Although not seen in the analysis of starvation resistance in chapter 2, females have greater starvation-resistance in other starvation-selected populations (Chippindale et al., 1996) and after more generations of selection in our populations a difference in starvation resistance between the sexes may become significant. An important difference between starvation-selected and fed control females is what happens to lipid content during adulthood (Figure 3.1). In fed control females the lipids are gained, but in starvation-selected females lipids are lost. It is difficult to explain how this might be possible, except that the massive amount of lipids that the starvation-selected females have upon eclosion, significantly more than any other fly group, cannot be maintained as an adult.

The purpose of investigating interactions usually is to build a model that can predict the value of one variable based on the known values of other variables. With these experiments not being originally designed for this purpose, only some possible relationships can be elucidated with reasonable certainty. These more certain relationships have been described above, however less conclusively, the analyzed interactions show support for a model indicating a trade-off between energy resources that may be related to the availability of physiological space. For example, starvation-selected females lose lipid mass while gaining glycogen and body mass (Figure 3.1-2). This suggests that a maximum of lipid content is reached and cannot be maintained even with an increase in body mass. By contrast an increase in glycogen, which occurs in all flies during adulthood, still occurs in starvation-selected female flies, suggesting that a minimum of glycogen content must be reached even at the possible cost or trade-off of

lipids. Starvation-selected flies also do not accumulate more protein with adult age, but fed control flies do (Figure 3.3), indicating that a minimum of protein is already accumulated and since it is not an energy resource for resisting starvation, more is not accumulated in the starvation-selected flies. Fed control flies increase their lipid, glycogen, and protein content with adult age implying that no maximum in any one energy resource type has been reached and no trade-off between energy resources occurs.

Although there is evidence indicating a trade-off between energy resources, further experiments must be designed to explicitly show relationships between different energy resource types and starvation resistance. This could be achieved by manipulating energy resource level, either by diet as adults/larvae, and then test for starvation resistance. Larger sample sizes would also be needed to test for interactions, because the more variables in the statistical model the lower the models' ability to detect significant differences and interactions.

Despite these shortcomings in support of my suggested model, it is still important to point out what the evolutionary implications in these starvation-selected populations would be if further evidence was found to support the model. A trade-off in energy resources at 47 generations of starvation selection implies an order, selection pressure, and generation number to achieve different starvation resistance strategies. At 47 generations of selection, there is evidence that maximum lipid accumulation may have been achieved, suggesting that subsequent generations may no longer be able to increase energy stores to increase starvation resistance (Figure 1.2 A). This will increase the selective pressure for other starvation-resistant strategies (Figure 1.2 B&C). Considering in chapter 2 activities levels tended to be lower in starvation-selected flies, but not

significant (Figure 2.8), my model would predict that the pressure for this starvation resistant strategy would increase and after more generations would be significant. Since energy storage will first reach its maximum as a resistance strategy, it suggests that energy storage is a highly plastic trait that would show the first signs of selection when first beginning starvation selection on a fly population. This is supported by the universally seen increase in lipid content in other populations of starvation-selected *Drosophila* (Baldal et al., 2006; Chippindale et al., 1996; Griffiths et al., 2005; Harshman, Hoffmann et al., 1999). My model also suggests that starvation resistance strategies will not change until selective pressure begins to shift from energy storage to energy usage strategies (Figure 1.2 A&B) and this will not occur in early generations of selection, because the energy resource storage maximum has not been reached. This is further supported in the literature by the lack of consistent results in other studies showing decreasing metabolic rates in populations under starvation selection (Baldal et al., 2006; Djawdan et al., 1997; Harshman, Hoffmann et al., 1999; Harshman & Schmid, 1998). Still further support is that total mass metabolic rates shown to conclusively decrease in selected populations, had undergone starvation selection for more than 60 generations (Djawdan et al., 1997; Harshman, Hoffmann et al., 1999). In other studies with 20 or less generations of selection, no conclusive decrease in total mass metabolic rates were seen (Baldal et al., 2006; Harshman & Schmid, 1998). My energy resource trade-off model, based on the analysis of energy resource interactions, suggests an evolutionary order to the development of starvation resistance in starvation-selected populations of *Drosophila*. Although the model is incomplete and requires further experimental investigation, nothing currently in the literature or in my findings dispute it.

CHAPTER 4

DEVELOPMENT CHANGES

4.1 Development, Lipid Content, and Starvation Resistance

In the previous chapter energy content data were teased apart into the categories of adult age, sex, and selection treatment with regard to how body mass was related to each. The results helped contribute to a broader understanding of how starvation resistance evolved in the starvation-selected flies. One of the central conclusions from chapter 2 and 3 was that lipids contribute to a large portion of the total body mass of our starvation-selected flies (Figure 3.1), and lipids are important for starvation resistance (Figure 2.2). The majority of lipids in the starvation-selected flies are accumulated during larval development, because the lipid stores are immediately present upon eclosion (Figure 3.1). Increased lipid content is a common way for drosophilids to increase their starvation resistance when under selection (Hoffmann & Harshman, 1999). Lipid content has been correlated with starvation resistance in natural populations (Sisodia & Singh, 2010; van Herrewege & David, 1997), but not in every study (Hoffmann et al., 2001). Chippindale et. al. (1996) suggested the increased lipids are largely accumulated during an extended larval development period. However an extension in development does not have to be responsible for an increase in lipid content (Chippindale, Leroi, Saing, Borash, & Rose, 1997; Hoffmann, Hallas, Anderson, & Telonis-Scott, 2005). These examples suggest that although lipids are important for starvation resistance, the contribution of development time to lipid accumulation is variable. It is possible that larvae simply eat faster or metabolize lipids differently during development.

To understand how a change in development time might contribute to adult lipid content, some understanding of the mechanisms that regulate larval development must first be understood. Larval molting and puparium formation are controlled through a series of pulses of 20-hydroxyecdysone (20E), which have been reviewed several times (Britton & Edgar, 1998; Chown & Gaston, 2010; Mirth & Riddiford, 2007; Riddiford & Truman, 1993). The following is a very brief summary, much of which is based on work in *Manduca sexta*. α-Ecdysone is produced in the prothoracic gland, that is part of the ring gland. Its release is regulated by the release of the prothoracicotropic hormone (PTTH). Once released it is converted in peripheral tissues into the most biologically active hormone, 20-hydroxyecdysone (20E). Molting occurs when 20E is released in the presence of juvenile hormone (JH). However once the larval critical weight is achieved, the weight after which feeding no longer affects time to pupariation, JH levels decrease. After PTTH is released, triggering 20E increase, larval growth ceases. After a period of wandering, pupariation is initiated by the last larval PTTH and then 20E pulse. How *D. melanogaster* determine when they have reached critical weight is not entirely understood, however it has been shown that the size of the prothoracic gland, dependent on insulin signaling, may be involved with sensing when critical weight is achieved (Britton & Edgar, 1998; Caldwell, Walkiewicz, & Stern, 2005; Colombani et al., 2003; Mirth & Riddiford, 2007). Changes in the timing of these pulses could be responsible for changing larval development period.

In this chapter I investigate the contribution of larval development period to the accumulation of lipids, an important metabolic fuel for starvation resistance. Lipids can be accumulated during larval development through eating more quickly, eating for a

longer time, or being stored more efficiently, all of which will be further increased by a longer larval development period. In Chapter 2, I mentioned that development time is longer in the starvation-selected flies than in the fed control flies and in this chapter the data are presented. Here I investigate how lipids are accumulated and the contribution of development length to the accumulation of lipids. I suggest a possible mechanism for extending larval development length through changes in 20E titer timing, subsequently increasing lipid stores. Furthermore, I propose the starvation-selected fly populations fit a model of development time and body size proposed in *Manduca sexta*.

4.2 Methods

4.2.1 Larval Wet Masses

Adult flies from 48 generations of selection were placed in a small population container and presented with hard-agar grape plates every hour for 8 hours at 25°C. The first two collections were discarded and the following collections were used for determining development time. This pattern of hourly egg collection reduces the potential for collecting retained fertilized eggs, thereby reducing the variance in measured development time. In order to cover more time points during development two separate cohorts of eggs were collect 6 hours apart from each other. From each of these two cohorts sixty eggs per population were divided evenly between three vials; each population replicate had three vials with twenty eggs in them for each collection cohort. Three larvae per population were removed from each vial every 12 hours during the 3rd instar. A total of nine larvae were collected per selection treatment per time point. All larvae used were visually identified by their anterior spiracles as being 3rd instar.

Individual larval wet masses were recorded for each larva using a Cahn micro balance (±0.001 mg). As described in chapter 2, in order to prevent maternal and/or paternal effects in experiments, all flies used for experiments were one generation removed from this selection process.

4.2.2 Development Time

Collections of eggs from each fly population at 53 generations of selection were made on grape agar petri plates every hour for 8 hours at 25°C continuing as described above. The following day, plates were cleared of any hatched larvae 1 hour before the desired hatching time. After the 1 hour period, forty-five recently-hatched larvae were collected and divided evenly between three vials; each population replicate had three vials with fifteen larvae in them for each collection time. The two collection cohorts 6 hours apart were checked every 12 hours for puparium formation and adult eclosion to cover more time points during development. As described in chapter 2, in order to prevent maternal and/or paternal effects in experiments, all flies used for experiments were one generation removed from this selection process.

4.2.3 Target gene expression profiles

Two collections of staged larvae were made 6 hours apart from one another. These larvae were staged from hatching the same way as those described in the development time assay. Larvae were placed in vials containing 10 ml of corn meal–sucrose–yeast medium. Larval collections were used to collect an RNA sample every 6 hours during the larval third instar (starting at 84 hours from egg lay until pupariation).

This was achieved by collecting RNA samples every 12 hours from both staged larvae collections. For each sample six larvae were washed and homogenized in a 1.5 ml tube containing 400 µl of TRIzol Reagent (15596-026, Invitrogen, Grand Island, NY, USA), then frozen at -80°C. All larvae used were visually identified by their anterior spiracles as being 3rd instar.

Nucleic acids were extracted from the samples preserved in TRIzol Reagent via the manufacturer's instructions from the Direct-zol RNA Miniprep Kit (R2052, Zymo Research, Irvine, CA, USA). The additional step of DNase removal of genomic DNA was included in the protocol. RNA was eluted from the columns with RNase/DNase free water, diluted to100 ng/µl and stored at -80°C. cDNA was made from the RNA samples by using the manufacturer's instructions for SuperScript First-strand cDNA Synthesis System for RT-PCR (11904-018, Invitrogen, Grand Island, NY, USA).

Genes of interest were selected with significant changes in RNA expression levels triggered by the last larval 20E pulse, associated with puparium formation (Andres, Fletcher, Karim, & Thummel, 1993; Arbeitman et al., 2002). Although many genes change RNA expression levels at this trigger, only those with very large shifts (a change of 2,000 or more on the modENCODE expression level scale) were used to increase the likelihood of visually determining presence and absence of bands on a gel. Ribosomal protein L23 (*RP L23*) was used as a reference gene not regulated by 20E, to show that all samples loaded on a gel contained similar cDNA levels. Genes of interest chosen and their designed primers are in Table 4.1. cDNA from genes of interest were amplified via PCR. The PCR mixture for each sample was comprised of 8 µl GoTaq Green Master Mix (M7122, Promega, Madison, WI, USA), 3 µl left primer (1 µM), 3 µl right primer (1

µM), and 1 µl cDNA (4 ng/µl). Following PCR, samples were loaded on a 1.5% TBE

Table 4.1

	Forward Primer	Reverse Primer
Ecdysone-induced protein 74A (E74A)	5’-TGT CCA TTC GCT TCT CAA TG-3’	5’-GTG CCA CCA AGC TGG AGT A-3’
Dopa decarboxylase (Ddc)	5’-AGT GAA GCC TGG CTA CCT GA-3’	5’-GGC ATG AAA CTT GGG ACT GT-3’
Urate oxidase (Uro)	5’-GTG GGC ACT CAC CTG AAG TT-3’	5’-GGA CTT TCA ATG CCA TGC TT-3’
Ribosomal protein L23 (RP L23)	5’-AGC CAA GAA CCT GTA CGT GA-3’	5’-GCA TGA CCT TCT TCC TGA GC-3’

Table 4.1 Primers: Primers used to amplify cDNA from genes of interest to determine timing of 20E pulse responsible for puparium formation. *Ribosomal protein L23* was used as a reference gene. See methods for details on selection method for other genes.

agarose gel and run at 120 volt for ~40 minutes and stained with ethidium bromide to visualize cDNA bands.

4.2.4 20-hydroxyecdysone Feeding Experiment

Eggs were collected on grape agar plates every two hours. These larvae were staged from hatching as described in the development time assay at 25°C. Two collection cohorts of larvae were made, 12 hours apart from one another. Approximately forty larvae were transferred from food vials 84 hours after egg deposition (AED) (or 60 hours from staged larval hatching plus 24 hours of embryogenesis) to 13x100 mm glass test tubes. Each tube contained 1 ml of standard fly food media with one of two solutions stirred well into the food; the controls contained 370 µl of 5% ethanol, and the 20E experimental treatment contained 370 µl 5% ethanol with 740 µg of 20-hydroxyecdysone (20E) (ALX-370-012, Enzo Life Sciences, Farmingdale, NY, USA). The 20E concentration in the 5% ethanol was 2 µg/µl; this is twice the concentration used to rescue ecdysone-deficient mutants before being mixed with food media (Klose, Gateff, Emmerich, & Beikirch, 1980). Previous pilot experiments indicated that the larger dose was necessary to induce pupariation. The final concentration of 20E in the food mixture was 0.54µg/µl. Test tubes where placed in a 25°C incubator and were monitored every 24 hours for formation of puparia and collection of eclosed adults. With two collection cohorts, 12 hours apart from one another, time points were collected every 12 hours.

4.2.5 Lipid Content and Eclosion Times

Adults eclosing from test tubes from the 20-hydoxyecdysone feeding were frozen at -20^{o}C upon eclosion and the time recorded. These flies were later thawed assayed for

total lipid content. Thawed flies were immediately placed in a 50°C oven for 24 hours before dry weights were recorded using a Cahn microbalance (±0.001 mg). Each fly was then placed into a small glass vial containing approximately 2 ml of diethyl ether for 24 hours. Flies were then removed from the ether and placed in a 50°C oven for 10 minutes and re-weighed. Lipid content was calculated by subtracting the weight after ether extraction from the original dry weight of the fly.

4.2.6 Statistics

Statistica 7 (Statsoft Inc., Tulsa, OK, USA) was used to run statistical models. Replicate populations were treated as random effects and nested within the selection treatment in all ANOVA models. Population replicates were variables in all models and were combined when effects are insignificant. Development time and larvae wet masses were analyzed with repeated measures ANOVAs. Pupariation time, lipid content, and eclosion time from 20E feeding experiment were analyzed with an ANOVA. Eclosion time vs. lipid content was then graphed from the least squared means and standard errors calculated from the ANOVAs to investigate interactions between the two variables.

4.3 Results

4.3.1 Larval Wet Masses

Larvae from both selection treatments, starvation-selected and fed control, gained wet mass significantly during 3rd instar ($p<0.001$) (Figure 4.1). However there was no significant difference in the rate of mass gained between the selection treatments ($p>0.05$) (Figure 4.1).

Figure 4.1

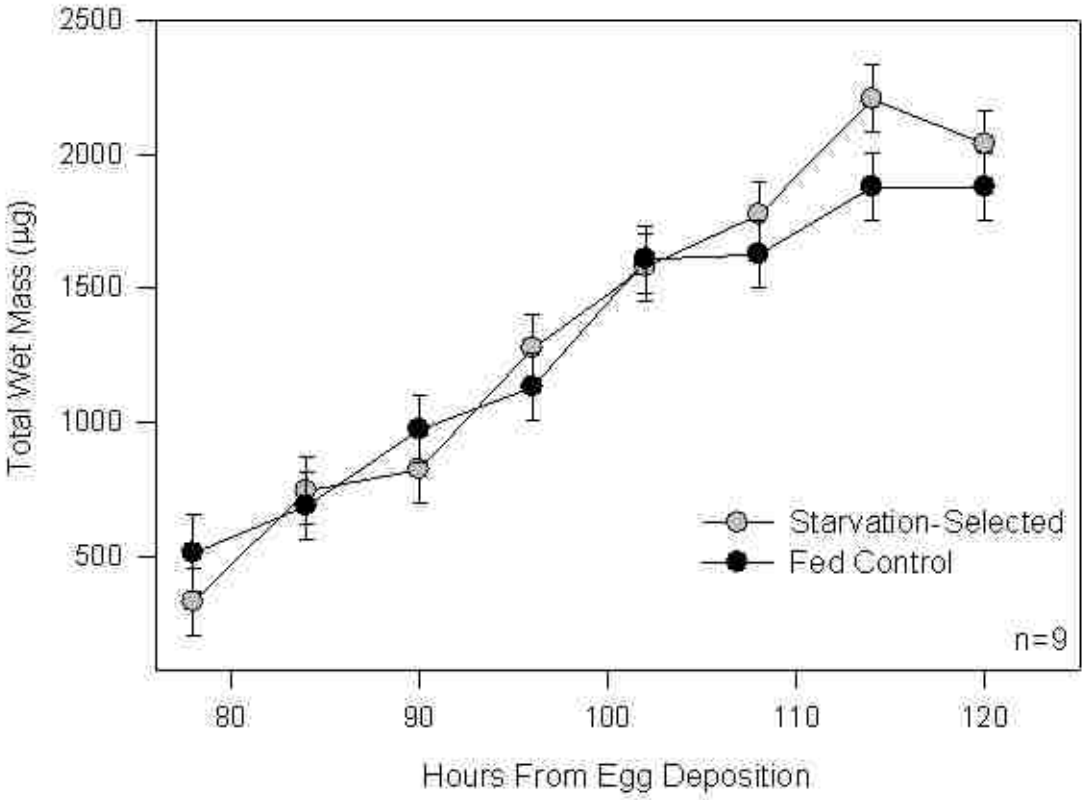


Figure 4.1 Larval wet mass: Least square means from repeated measures ANOVA of larval wet mass during the 3rd instar. Starvation-selected and fed control flies gained mass during the 3rd instar at the same rate ($p>0.05$). Error bars represent standard error.

4.3.2 Development Time

Larval development time was roughly 24 hours longer in starvation-selected flies than fed control flies at 53 generations of selection (Figure 4.2). Overall egg to adult development time was significantly longer in starvation-selected flies as well, but by roughly the same 24 hour difference was seen in the larval development period (Figure 4.3), indicating that the delay in development occurs mostly during larval development.

4.3.3 Target gene RNA expression profiles

The *RPL23* bands appeared equally intense for all lanes, indicating consistent cDNA levels and pipetting on gels (Figure 4.4 A&B) (Appendix B). The gene *E74A* is directly induced by 20E during the 20E pulse responsible for puparium formation and is not detected in samples before the pulse (Andres et al., 1993). All starvation-selected populations showed a delay in the expression of *E74A* RNA compared to when expression occurred in fed control flies (Figure 4.4 C&D) (Appendix B). The gene *Ddc*, although not reported to be induced by 20E, shows an accumulation in band intensities at late stages (Andres et al., 1993). All starvation-selected populations showed a delay in the increase in *Ddc* RNA expression compared to the timing of expression in fed control flies (Figure 4.5 A&B) (Appendix B). The gene *Uro*, like *Ddc*, changes RNA expression as a secondary response to 20E, its expression decreases in response to the 20E pulse. Although using gels to show decrease in RNA expression is less clean than looking for increases in expression, the starvation-selected populations still showed a delay in the decrease in expression when compared to their fed control counterparts (Figure 4.5 C&D) (Appendix B).

Figure 4.2

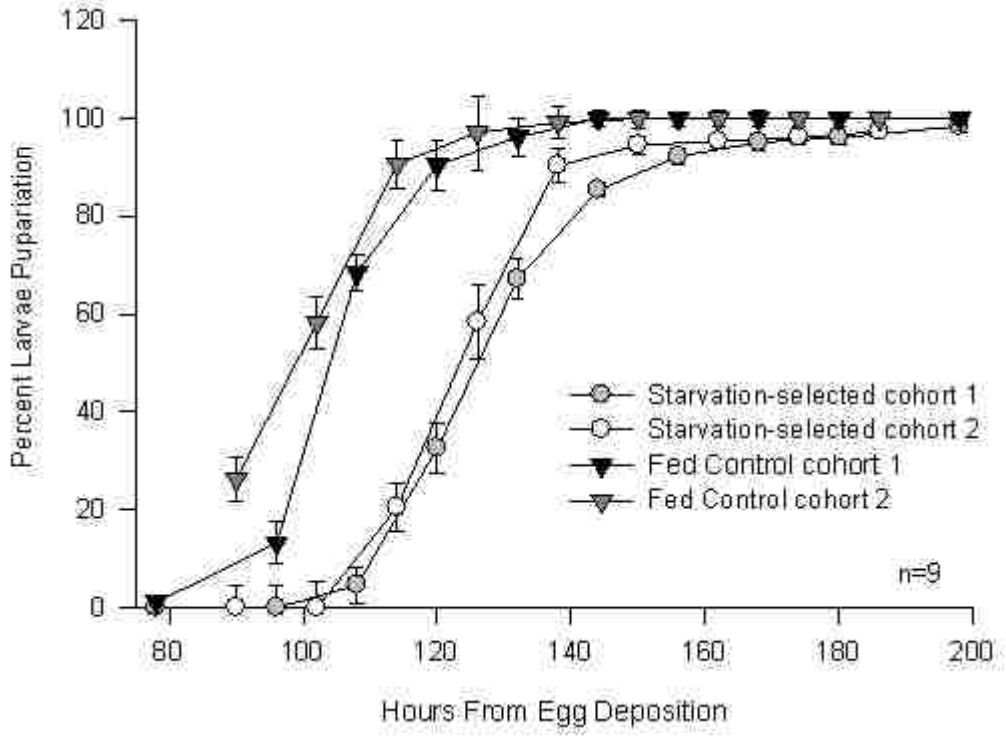


Figure 4.2 Larval development time: Least square means from repeated measures ANOVA of puparium formation percentages in vials. Two cohorts were used to cover more time points during development and are labeled separately. Starvation-selected flies had a significantly longer larval development time compared to fed control flies ($p<0.01$). Error bars represent standard error.

Figure 4.3

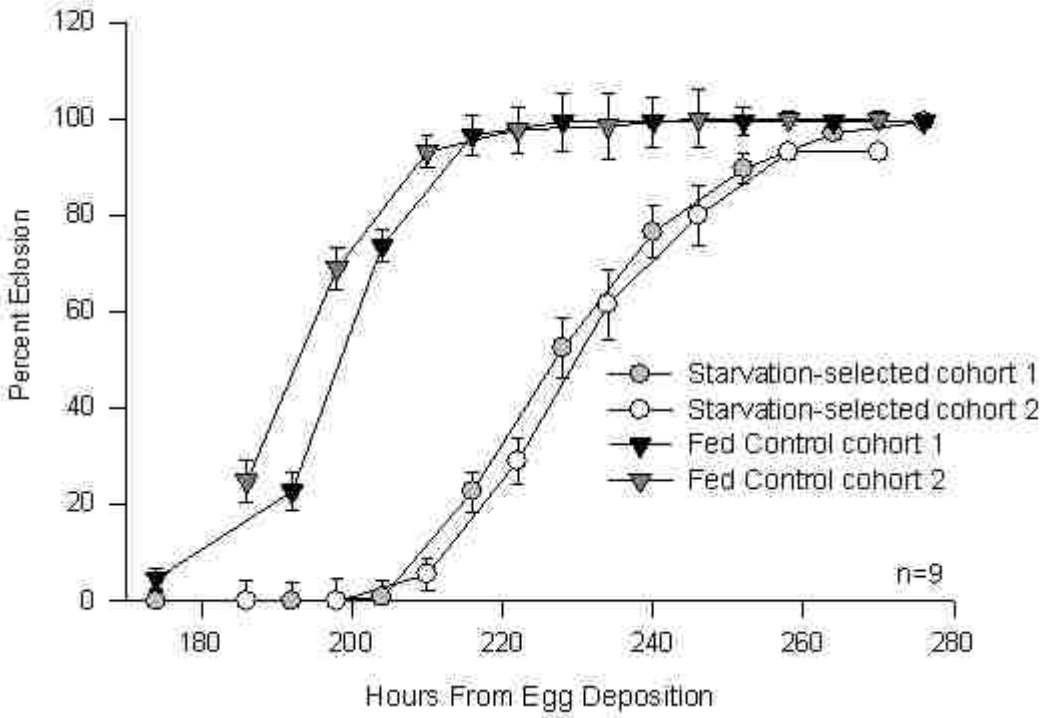


Figure 4.3 Eclosion time: Least square means from repeated measures ANOVA of eclosion time percentages in vials. Two cohorts were used to cover more time points during development and are labeled separately. Starvation-selected flies had a significantly longer egg to adult development time compared to fed control flies ($p<0.01$). Error bars represent standard error.

Figure 4.4

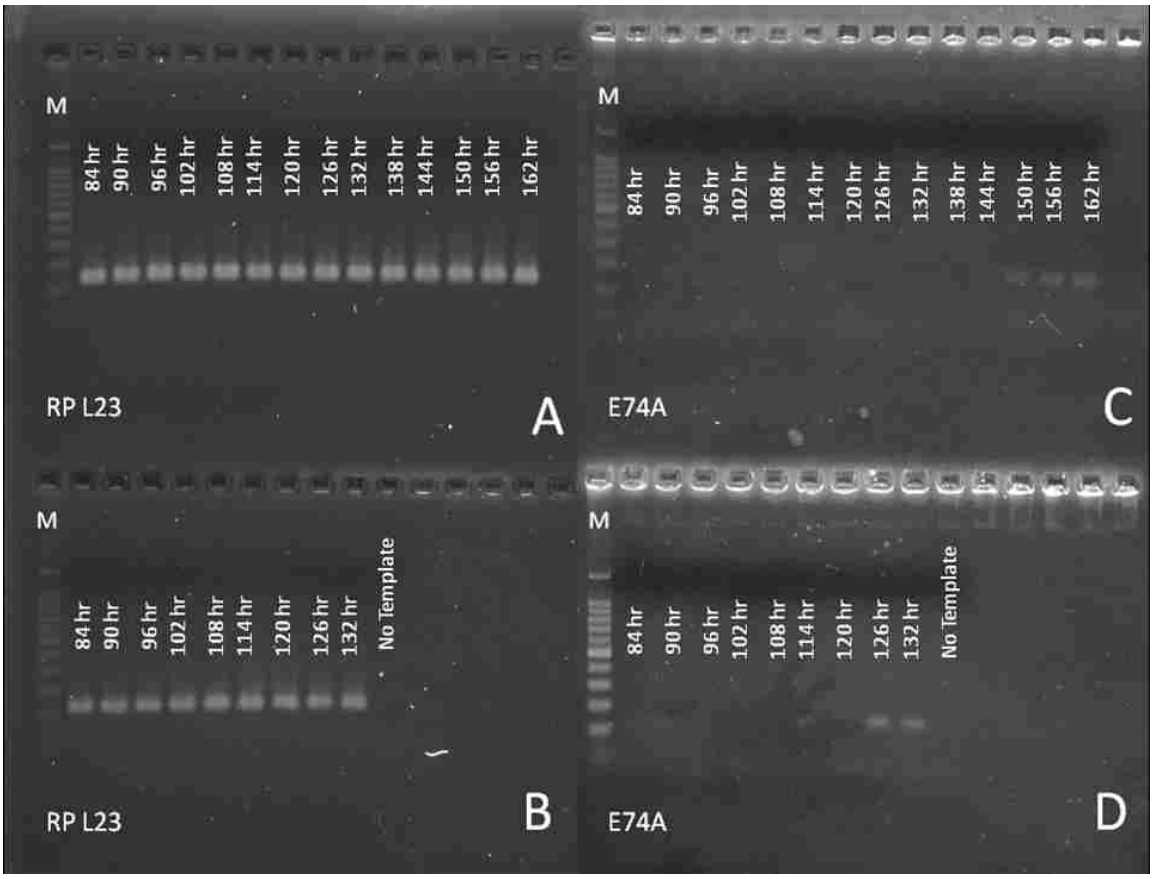


Figure 4.4 RNA levels for *RP L23* & *E74A*: cDNA amplified via PCR with primers from Table 4.1. Times represent hours from egg deposition. A) *RP L23* reference levels in starvation-selected fly replicate population C. B) *RP L23* reference levels in fed control fly replicate population C. C) *E74A* levels in starvation-selected population replicate population C. D) *E74A* levels in fed control fly replicate population C.

Figure 4.5

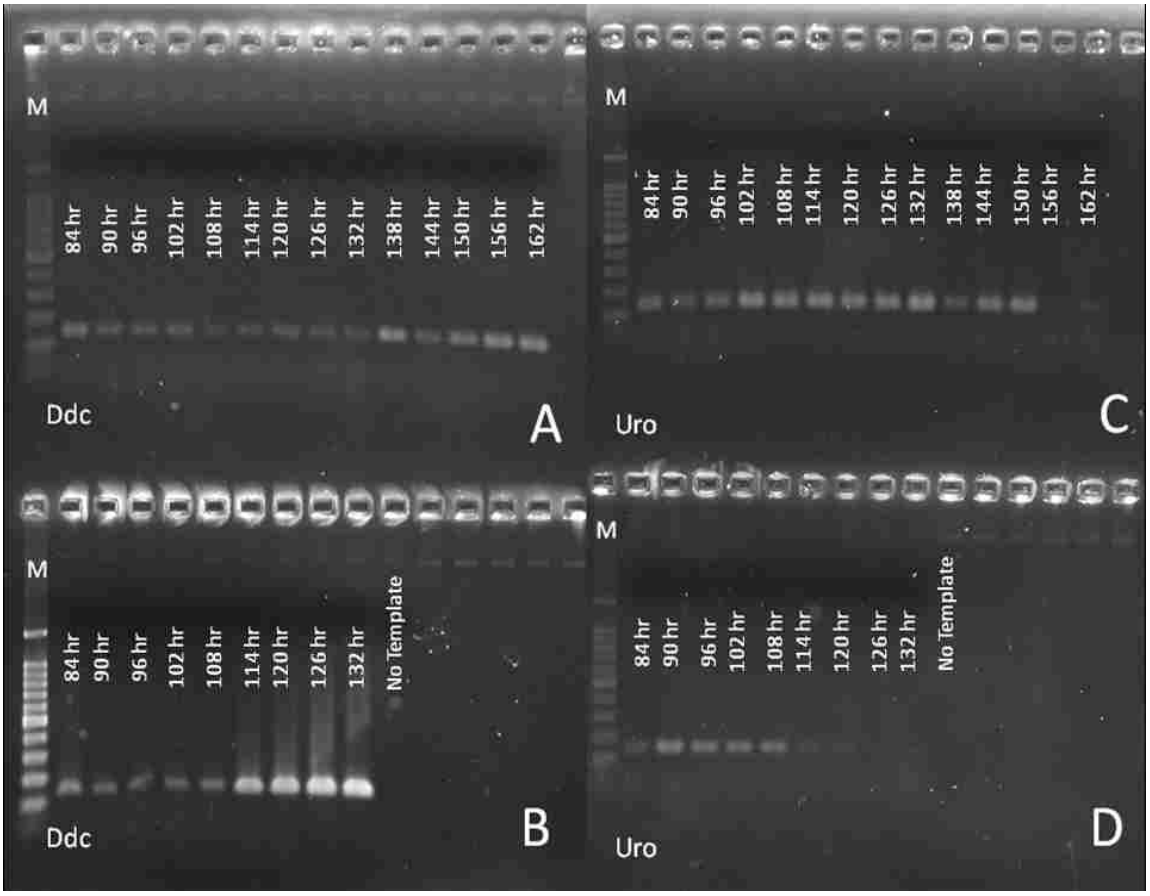


Figure 4.5 RNA levels for *Ddc* & *Uro*: cDNA amplified via PCR with primers from Table 4.1. Times represent hours from egg deposition. A) *Ddc* levels in starvation-selected fly replicate population A. B) *Ddc* levels in fed control fly replicate population A. C) *Uro* levels in starvation-selected fly replicate population A. D) *Uro* levels in fed control fly replicate population A.

4.3.4 20-hydroxyecdysone Feeding Experiment

Starvation-selected larvae fed 20E early in 3rd instar development, 3rd instar was positively identified via anterior spiracles, underwent pupariation approximately 75 hr earlier than their control starvation-selected counterparts ($p<0.001$). Fed control larvae also pupariated ~20 hr earlier when fed 20E ($p<0.001$) (Figure 4.6). Starvation-selected larvae fed 20E formed puparium later than fed control larvae fed or not fed 20E ($p<0.001$) (Figure 4.6). This indicates that although larval development was shortened by feeding 20E to the starvation-selected flies, their larval development length was not as short as the fed control flies.

4.3.5 Lipid Content and Eclosion Times

Data collected during the 20E feeding experiment made it possible to compare lipid content and development times within individual flies. Lipid content and eclosion time were positively correlated; indicating that as eclosion time increased so did lipid content (Figure 4.7). Newly eclosed starvation-selected flies fed 20E as larvae had significantly lower lipid content than starvation-selected flies not fed 20E ($p<0.001$). 20E fed starvation-selected flies also eclosed significantly earlier than their controls not fed 20E ($p<0.001$). However fed control flies fed 20E did not have significantly lower lipid content compared to their controls not fed 20E ($p>0.05$) even though they eclosed significantly earlier ($p<0.001$) (Figure 4.7).

Figure 4.6

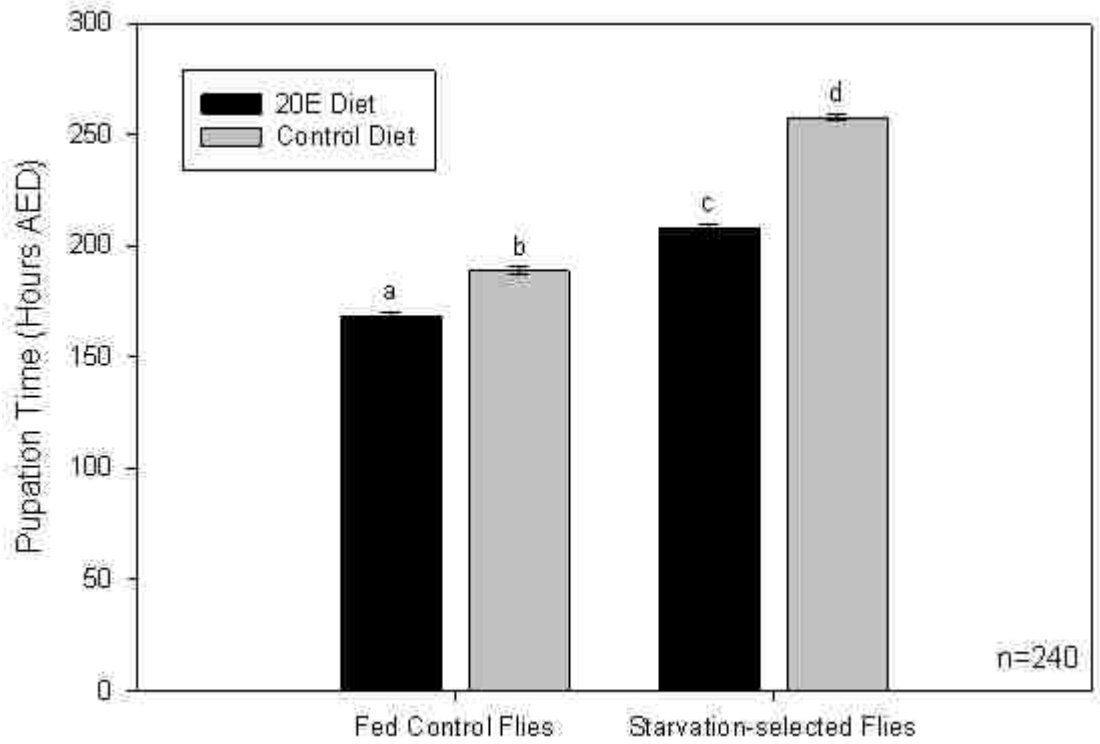


Figure 4.6 Pupariation times after 20E feeding: Least square means of individual larval pupariation formation times during the 20E feeding experiment. Flies fed the 20E diet pupariated significantly earlier than their control diet counterparts ($p<0.01$). Error bars represent standard error.

Figure 4.7

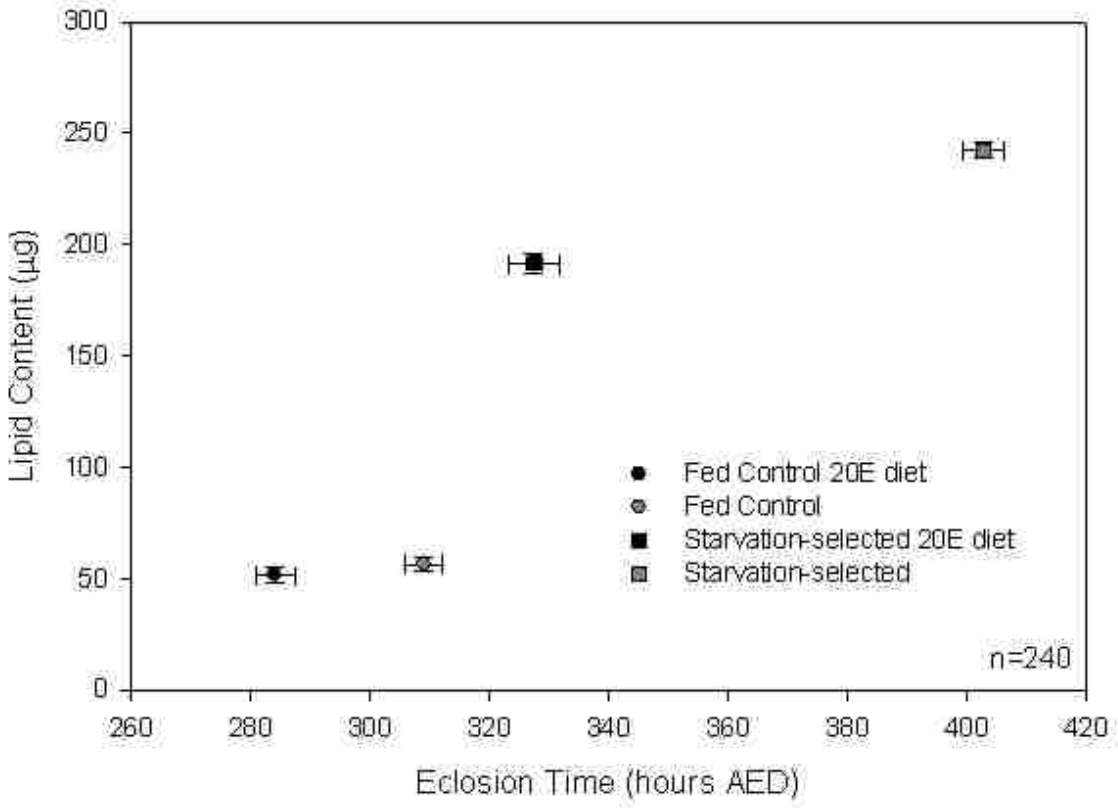


Figure 4.7 Eclosion time vs lipid content: Starvation-selected adult flies contain less lipid when fed 20E ($p<0.001$). When fed 20E, the timing of eclosion was significantly less in both the starvation-selected flies and fed control flies ($p<0.001$), however unlike in the starvation-selected flies there was no significant reduction in adult lipid content in the fed control flies ($p>0.05$). Least square means with error bars representing standard error.

4.4 Discussion

In chapters 2 and 3 I showed that lipids were important for starvation resistance (Figure 2.2) and they were a large portion of the total body mass of starvation-selected flies (Figure 3.1). These lipids were also accumulated during larval development (Figure 3.1). At generation 53 the starvation-selected flies had roughly a 24 hour extension in larval development compared to the fed control flies (Figure 4.2). In some cases flies selected for starvation resistance have increased their development time (Chippindale et al., 1996; Harshman, Moore, Sty, & Magwire, 1999) and in other cases they have not (Hoffmann, Hallas et al., 2005). However these extensions in development time did not exceed 5-10 hours (Chippindale et al., 1996; Harshman, Moore et al., 1999), making the 24 hour extension in our populations a unique case. This is likely due to the fact that my fly populations were allowed to develop in bottles until nearly all flies had eclosed, whereas other studies restricted overall development to 14 days (Chippindale et al., 1996). Chippindale et. al. (1996) suggests the increased lipids are largely accumulated during an extended larval development period. Evidence from my work supports this idea, since during 3rd instar development, total wet body mass increased at the same rate between the selection treatments (Figure 4.1).

The delayed development phenotype may play an important role in the development of starvation-resistance in adults, either by contributing to increased lipid stores or larger total body mass. To investigate this developmental extension, I investigated the possibility of a delay in the hormone cascade responsible for the last larval 20E pulse to initiate puparium formation. Several genes whose expression pattern are known to be regulated by 20E were investigated (Andres et al., 1993; Arbeitman et

al., 2002) (Table 4.2). The genes *Ddc* and *E74A* both had undetectable expression levels of RNA before this 20E pulse, but increased substantially following the pulse. This expression change occurred roughly 24 hours later in the starvation-selected flies (Figure 4.4 C&D and Figure 4.5 A&B). The *Uro* gene RNA levels visibility decreased in response to this 20E pulse and this decrease occurred 34 hours later in the starvation-selected flies (Figure 4.5 C&D). These time gaps in gene RNA expression correspond well with the approximately 24 hour extension in development seen in the starvation-selected flies. All population replicates showed a similar gap in the timing of their gene RNA expression changes that correspond with their respective development times (Appendix B). Although all population replicates showed the same pattern of delay, some variation in the length of the delay occurred between population replicates (Appendix B); indicating that many genes and alleles are involved in regulating the hormonal cascade. The change in the timing of gene RNA expression shifts supports the idea that there is a delay in the last 20E pulse responsible for pupariation. This is not, however, conclusive evidence of a shift in the 20E pulse, only supporting evidence. An alternative hypothesis to the developmental delay would be that the larval response to 20E hormonal pulses is altered. Further experiments distinguishing these hypotheses are still needed. These further experiments should investigate the many possible mechanisms of the developmental delay such as a delay in the final PTTH pulse, delayed expression of the 20E receptor (EcR & USP), or a more gradual accumulation of 20E levels in the starvation-selected populations. An actual quantitative measure of hormonal levels of JH, PTTH, and 20E would be a better approach to determining mechanism; however my data

do provide a beginning for determining when during development samples for measuring hormonal levels should be conducted.

The starvation-selected flies may have delayed larval regulatory hormone expression and their response to an increase in 20E earlier than their delayed timing would indicate if the appropriate mechanisms for responding to 20E are present earlier in 3rd instar. The starvation-selected and fed control flies were fed 20E early in 3rd instar, to mimic the 20E pulse responsible for pupariation, with the intention of shortening larval development. As predicted, all fly populations fed 20E formed puparia significantly earlier than their experimental controls (Figure 4.6). This experiment indicates that mechanisms for detecting and responding to the 20E pulse in the starvation-selected flies are functional earlier in development. However despite being fed 20E at the same time as the fed control flies, the starvation-selected flies never shortened their larval development time to the same level as the fed control flies. This experiment does not indicate why this might be, but allows for some speculation that appropriate levels of JH, PTTH, and/or 20E have not been achieved until after the same time period in the fed control flies. It can also be speculated that the 20E receptor (EcR &USP) could be at insufficient levels to drive pupariation at the same time as the fed control flies, but within 20 hours of pupariation in the fed control flies, the starvation-selected flies reached sufficient receptor levels. Only through further experimentation can these details be worked out. All flies however, took longer than usual to develop when compared to the development time assay from generation 53 (Figure 4.2 & 4.6). This is not entirely unexpected with larvae being washed and moved to new media during third instar. Such disturbances and handling have been known to delay normal development (Ashburner, 1989), however

because all larvae were treated the same, possible effects on the experiment are minimized. Even with all these caveats, these experiments support the hypothesis that the longer development period in the starvation-selected flies is due to changes in normal hormonal regulation.

A positive correlation between development time and starvation resistance is commonly seen in laboratory starvation-selected *Drosophila* populations (Gibbs & Reynolds, 2012; Rion & Kawecki, 2007), but not seen in natural populations (van der Linde & Sevenster, 2006). The difference in development time between the starvation-selected flies and their fed controls was mostly confined to larval development; the same 24 hour delay was seen in both puparium formation and eclosion times (Figure 4.2-3). This makes a comparison between eclosion times and lipid content very similar to comparing larval development time to lipid content. Lipid content and eclosion times were positively correlated; indicating that overall a greater eclosion time is connected with greater adult lipid content (Figure 4.7). This positive correlation confirms that development time, lipid content, and starvation resistance are related (Chippindale et al., 1996; Harshman, Hoffmann et al., 1999). However the connection alone does not indicate how much larval development time contributes to increasing lipid stores. By shortening the larval development period through feeding 20E and measuring how lipid content changes, this can be investigated.

As previously seen with timing of pupariation (Figure 4.6), the timing of adult eclosion was significantly reduced when larvae were fed 20E in both starvation-selected and fed control flies ($p<0.01$) (Figure 4.7). The reduction in eclosion time was three times greater however in starvation-selected flies (Figure 4.7). This greater difference in

starvation-selected flies likely contributes to their significant decline in total adult lipid content ($p<0.01$) (Figure 4.7) thereby connecting development time directly to lipid content in starvation-selected flies. The smaller, but still significant reduction in development time in the fed control flies with 20E feeding ($p<0.01$) did not result in a significant reduction in adult lipid content ($p>0.05$) (Figure 4.7), indicating that the smaller change in development time was insufficient to reduce lipid content or that lipid content and development time do not have the same relationship seen in starvation-selected flies. The latter idea is further supported by the large difference in lipid content seen between fed control flies not fed 20E and starvation-selected flies fed 20E ($p<0.01$), despite a relatively small difference between their eclosion times ($p=0.03$) (Figure 4.7). Starvation-selected flies fed 20E shortened their development by nearly 75 hours, but their lipid content was reduced by only 50µg. This striking result indicates that differences in lipid accumulation during the third instar are not simply a result of different development times.

Starvation selection in *Drosophila* commonly increases development time (Gibbs & Reynolds, 2012; Rion & Kawecki, 2007). Yet in my starvation-selected flies an extended larval development period only accounted for a small proportion of the lipids accumulated during development (Figure 4.7). So why does development time form a positive correlation with starvation resistance under starvation selection and yet vary in relation to starvation resistance in natural populations of *Drosophila* (van der Linde & Sevenster, 2006)? Insight into the evolutionary mechanisms responsible for selecting for a change in larval developmental period in starvation-selected flies may contribute to understanding the variation and how starvation resistance develops in *Drosophila*.

Models proposed in the tobacco hornworm, *Manduca sexta*, investigating development time and body size already provide some insight into this evolutionary conundrum. Nijhout et. al. (2010) uses the extensive knowledge of *M. sexta* growth and development regulation, much of which is shared by *D. melanogaster*, to predict the relationship between development period and body size based on different developmental and physiological parameters, which are subject to natural selection. Their findings suggest all feasible correlations between body size and development period are possible (Nijhout et al., 2010). Further adaptation of this model by Davidowitz et. al. (2012) included a genetic architecture for the developmental and physiological parameters affecting correlations between development length and body size. This new genetic architecture predicts that when selection in the same direction on body size and development time occur (both increasing or decreasing) the underlining mechanism correlating the two life history traits is most often the result of changes in hormonal regulation of development time (Davidowitz, Nijhout, & Roff, 2012). However when selection acts on development time and body size in opposite directions (one increases and other decreases) the underlying mechanisms correlating the two life history traits most often rely on changes in growth rates (Davidowitz et al., 2012). Relating this to the starvation-selected flies, earlier in this chapter a correlation between development time and adult lipid content was seen that varied in significance between starvation-selected and fed control flies (Figure 4.7). From chapter 3 a correlation between lipid content and body mass, one indicator of body size, was also seen (Figure 3.1). Together these give some indication of the relationship between body size and development length in the starvation-selected flies, a positive correlation with both body size and development length increasing. The model

created in *M. sexta* would predict from this type of correlation a change in hormonal regulation of development length in the starvation-selected flies, but no change in their larval growth rates compared to fed control flies (Davidowitz et al., 2012). As the model predicts, the starvation-selected flies have shown evidence of a change in hormonal regulation resulting in increased larval development (Figure 4.4-5) as well as no change in the rate of wet mass accumulation during 3rd instar (Figure 4.1). The implication that this model might be capable of predicting relationships between body size and development in *D. melanogaster*, creates new possibilities for explaining the variations in these characteristics seen in *D. melanogaster* populations.

From the investigations of this chapter I have found that the starvation-selected flies likely delay their larval development through changes in hormone regulation. This is indicated by delays in gene RNA expression changes associated with the 20E pulse responsible for puparium formation and shortening the larval development time through feeding of the hormone 20E. By manipulating larval development length, I was able to discover that although development length contributes to lipid accumulation, it is not the only mechanism used to increase lipid stores during development. Lipid metabolism also must play a role. The indication of a relationship between development time and lipid content in starvation-selected flies suggests a relationship also with body size. An evolutionary model developed in *M. sexta* is thus able to be adapted and show potential for predicting selective pressures in starvation-selected *D. melanogaster*.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Populations of organisms are continually evolving under environmental pressures all around us. Comprehension of this process allows us to better understand the world in which we currently live and predict where it will be in the future. Due to the commonality of the environmental pressure of starvation and how many organisms face a life stage with limited food, starvation resistance and how it is achieved is of particular interest. *Drosophila* have a long scientific history, and more is known about their response to starvation than in any other insect. They also offer a unique perspective on starvation resistance due to the wide range of studies completed on *Drosophila* and related species. Their ease of maintenance, short generation span, and genetic resources are a few of the reasons *Drosophila* have been brought into laboratories for experiments in selection for starvation resistance. Possible mechanisms for surviving periods of starvation are summarized in a Figure 1.2: increasing energy stores, decreasing rate of energy depletion, decreasing level of energy to maintain life. In populations of *Drosophila melanogaster* selected for starvation resistance evidence of an increase in energy storage is common, yet no consensus on how metabolic rates change (and therefore energy usage) has been found, and no one has attempted to examine the energy levels required to maintain life (Gibbs & Reynolds, 2012; Rion & Kawecki, 2007). Despite all of the studies on *Drosophila* no one study has examined all of these strategies on a single population set of *Drosophila*. Selection in natural populations is variable (Gibbs, 1999; Gibbs & Gefen, 2009) and comparisons between very different groups of

Drosophila make it impossible to elucidate the finer details of how life history traits interact and contribute to these survival strategies. For my dissertation I characterized all of these starvation resistant strategies in a single group of starvation-selected *D. melanogaster*, allowing me to use the relationships between the life history traits measured to test further hypotheses on the evolution of starvation resistance.

The starvation-selected *D. melanogaster* populations that I investigated have achieved increased starvation resistance through a combination of two mechanisms; lipids are preferentially stored for energy (Figure 2.2) and decreased metabolic rates conserve their stored energy (Figure 2.7). Lowered energy limit to maintain life, the third strategy, is not seen (Figure 2.2-5). These strategies have a marked trade-off with decreased fecundity (Figure 2.6) and longer development time (Figure 4.2) in the starvation-selected flies. This detailed characterization of life history traits allowed me to further investigate interactions between energy storage and body mass. These interactions emphasized the importance of lipids as an energy source for resisting starvation and provided evidence of a possible trade-off in the types of energy stored (Figure 3.1-3). Further validation of this possible trade-off is needed through careful dietary studies, but the evolutionary implications of such a trade-off provide some idea of a shift in starvation resistance strategies. As energy storage increases under starvation selection, a shift may occur from increased energy storage to decreasing rates of energy usage (Figure 1.2 A&B). This could explain why energy storage is common, but changes in metabolic rates are less common in other starvation-selected *Drosophila* (Gibbs & Reynolds, 2012; Rion & Kawecki, 2007); if energy storage has not reached a maximum, the selective pressure for a change in metabolic rates will not be as strong. This implies also an order to the

starvation resistant strategies, first energy storage followed by decreasing rate of energy usage (Figure 1.2 A&B). A future direction of study would be to investigate if these strategies actually evolve in this order.

Most lipid was accumulated during larval development (Figure 3.1), indicating the increase in development time is not just a trade-off, but may contribute to starvation resistance through increasing the time during which an increase in lipid mass and body mass can occur. This has been suggested before (Chippindale et al., 1996), but how much development time contributes to larval lipid accumulation has not been investigated until now. When larval development time is significantly shortened, lipid content in the starvation-selected flies also significantly decreases (Figure 4.4). This confirms that development length does contribute to lipid accumulation; however development length does not entirely account for the increased lipid content. In fact, when fed control flies have a similar development length compared to the shortened development of starvation-selected flies, the lipid content in the starvation-selected flies is still three times that of the fed control flies (Figure 4.4). This disparity in lipid content indicates that lipid metabolism differs substantially between the starvation-selected populations and their controls.

The mechanism by which the starvation-selected flies extend their development is consistent with a recent evolutionary model developed for the relationship between body size and development length in *Manduca sexta* (Davidowitz et al., 2012). This model predicts that when there is positive selection on development time and body size a change in the hormonal mechanism regulating the length of larval development occurs; when there is opposing selection between development time and body size a change in larval

growth rates occurs (Davidowitz et al., 2012). The starvation-selected flies show a delay in the RNA expression of genes associated with a regulatory hormonal pulse during larval development (Figure 4.5-6), consistent with positive selection for both development time and body size. Further experiments to corroborate a change in hormonal regulation are needed, but this preliminary evidence supports the model and its use for study of evolution in *Drosophila* populations. Positive selection on both of these traits could indicate that development time might also contribute to a larger overall body size. One way to prevent reaching a maximum in energy storage could be have a larger body size, providing another reason for extending larval development time. An experiment investigating when critical weight is achieved and how much growth is continued beyond this point will give further insights into regulation of body composition and body size during the delayed development of the starvation-selected flies.

By completing my investigation into characterizing the starvation resistance strategies of starvation-selected *D. melanogaster*, I have discovered many new insights into the evolutionary mechanisms of starvation resistance and opened new areas for further investigation. A combination of energy storage and slow energy usage contribute to starvation resistance in these starvation-selected flies. The comparative analysis of the relationships between energy stores and body mass indicates the possibility of trade-offs between energy stores and suggests an order and direction of starvation resistant strategies and selective pressures. By examining development time and its contribution to lipid stores in individual flies, I have overturned the hypothesis that a delay in development is solely responsible for an increase in adult lipid content. Lipid metabolism must also contribute significantly to larval lipid accumulation. Delayed development may

also have a positive relationship with body size, contributing to increasing the energy storage for resisting starvation and supporting the application of a model developed for *Manduca sexta* in *Drosophila*.

APPENDIX A ANOVA TABLES

ANOVA tables for figures in chapter 3

Lipid Mass (µg)	degrees of freedom	F	p
Intercept	1	636.409	0.000015
Selection	1	186.665	0.000166
Population (Selection)	4	1.118	0.461888
Sex	1	13.644	0.020949
Age	1	0.422	0.551335
Population (Selection)*Age	4	1.266	0.412501
Population (Selection)*Sex	4	3.529	0.124707
Selection*Age	1	43.694	0.002715
Selection*Sex	1	0.009	0.929315
Sex*Age	1	0.233	0.654671
Selection*Sex*Age	1	7.697	0.050108
Population (Selection)*Sex*Age	4	0.872	0.484030
Error	96		

Glycogen Mass (µg)	degrees of freedom	F	p
Intercept	1	525.204	0.000021
Selection	1	2.479	0.190499
Population (Selection)	4	1.124	0.450472
Sex	1	11.218	0.028588
Age	1	114.020	0.000436
Population (Selection)*Age	4	12.903	0.014777
Population (Selection)*Sex	4	1.618	0.326158
Selection*Age	1	0.503	0.517391
Selection*Sex	1	29.153	0.005695
Sex*Age	1	10.142	0.033387
Selection*Sex*Age	1	4.943	0.090276
Population (Selection)*Sex*Age	4	0.085	0.986821
Error	96		

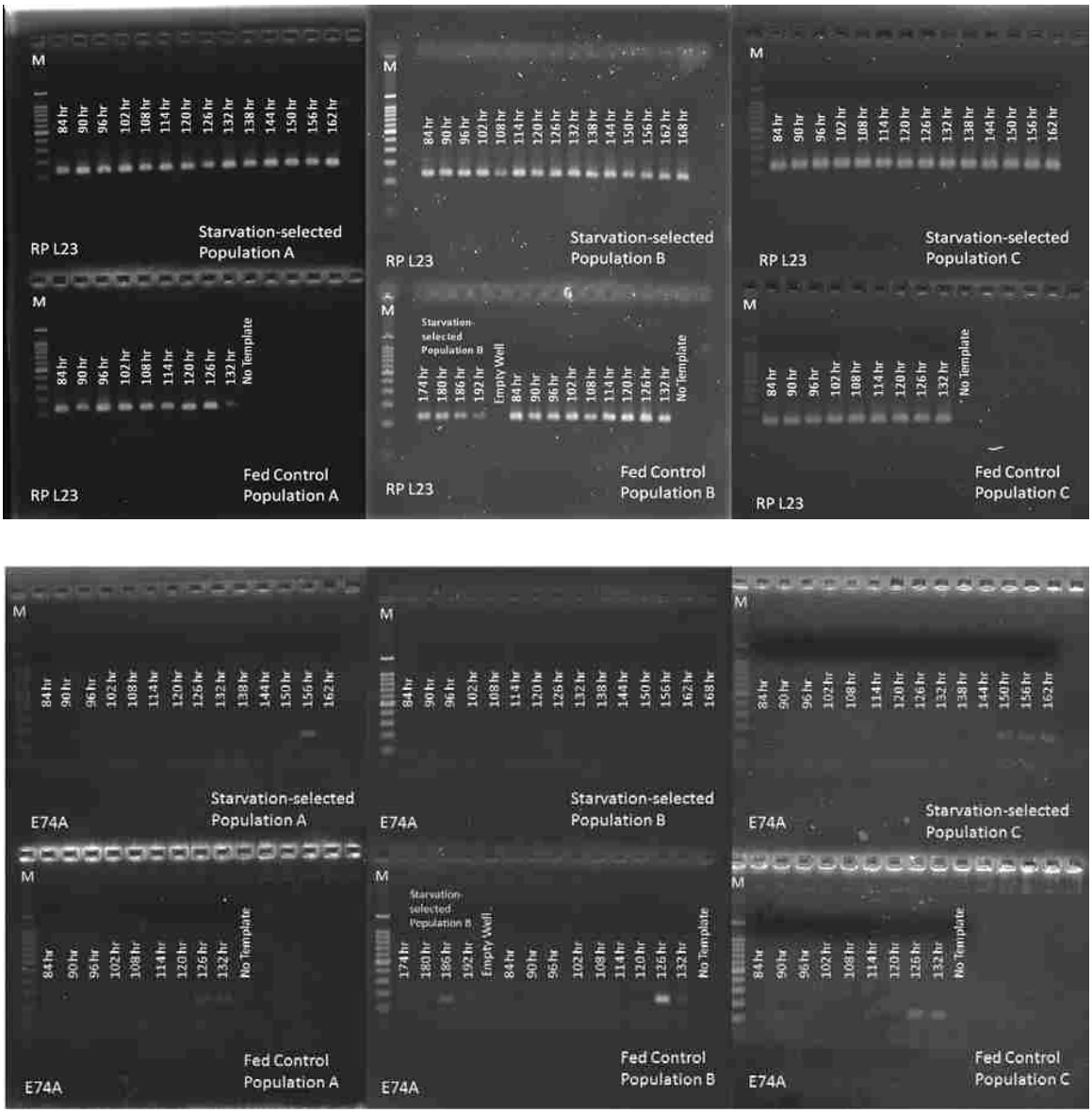
Protein Mass (µg)	degrees of freedom	F	p
Intercept	1	5312.370	0.000000
Selection	1	0.081	0.790628
Population (Selection)	4	1.275	0.415967
Sex	1	0.166	0.704333
Age	1	37.286	0.003640
Population (Selection)*Age	4	4.269	0.094396
Population (Selection)*Sex	4	1.108	0.461760
Selection*Age	1	6.508	0.063239
Selection*Sex	1	72.501	0.001044
Sex*Age	1	20.614	0.010495
Selection*Sex*Age	1	0.104	0.763718
Population (Selection)*Sex*Age	4	0.055	0.994260
Error	96		

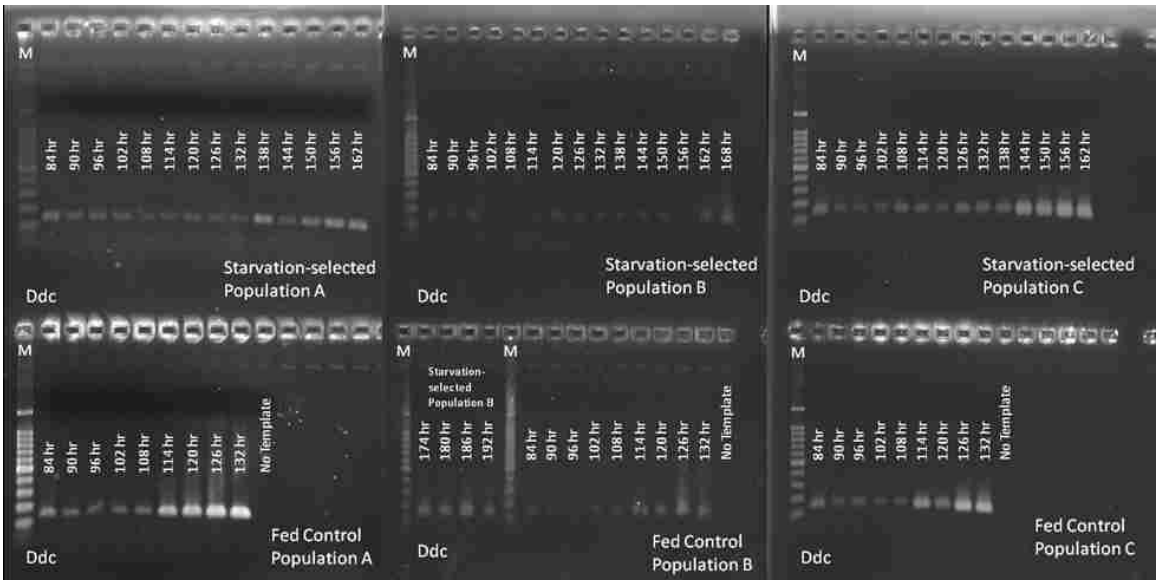
Total Body Wet Mass for Lipid (µg)	degrees of freedom	F	p
Intercept	1	2544.450	0.000001
Selection	1	37.161	0.003663
Population (Selection)	4	1.779	0.302528
Sex	1	120.196	0.000393
Age	1	12.632	0.023711
Population (Selection)*Age	4	1.097	0.465379
Population (Selection)*Sex	4	4.524	0.086461
Selection*Age	1	0.660	0.462200
Selection*Sex	1	0.747	0.436288
Sex*Age	1	26.268	0.006861
Selection*Sex*Age	1	0.281	0.623956
Population (Selection)*Sex*Age	4	0.682	0.606425
Error	96		

Total Body Wet Mass for Glycogen and Protein (µg)	degrees of freedom	F	p
Intercept	1	4936.083	0.000000
Selection	1	65.838	0.001254
Population (Selection)	4	0.420	0.789398
Sex	1	81.215	0.000840
Age	1	2.808	0.169095
Population (Selection)*Age	4	4.167	0.097857
Population (Selection)*Sex	4	9.939	0.023542
Selection*Age	1	0.540	0.503278
Selection*Sex	1	0.114	0.752548
Sex*Age	1	122.719	0.000378
Selection*Sex*Age	1	5.212	0.084516
Population (Selection)*Sex*Age	4	0.535	0.710075
Error	96		

APPENDIX B cDNA GELS

cDNA gels for all replicate populations from chapter 4

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126 hr
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Starvation-selected Population A
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Ddc
Fed Control Population A
M
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102 hr
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114 hr
120 hr
126 hr
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150 hr
156 hr
162 hr
168 hr
Ddc
Starvation-selected Population B
M
Starvation-selected Population B
174 hr
180 hr
186 hr
192 hr
M
84 hr
90 hr
96 hr
102 hr
108 hr
114 hr
120 hr
126 hr
132 hr
No Template
Ddc
Fed Control Population B
M
84 hr
90 hr
96 hr
102 hr
108 hr
114 hr
120 hr
126 hr
132 hr
138 hr
144 hr
150 hr
156 hr
162 hr
Ddc
Starvation-selected Population C
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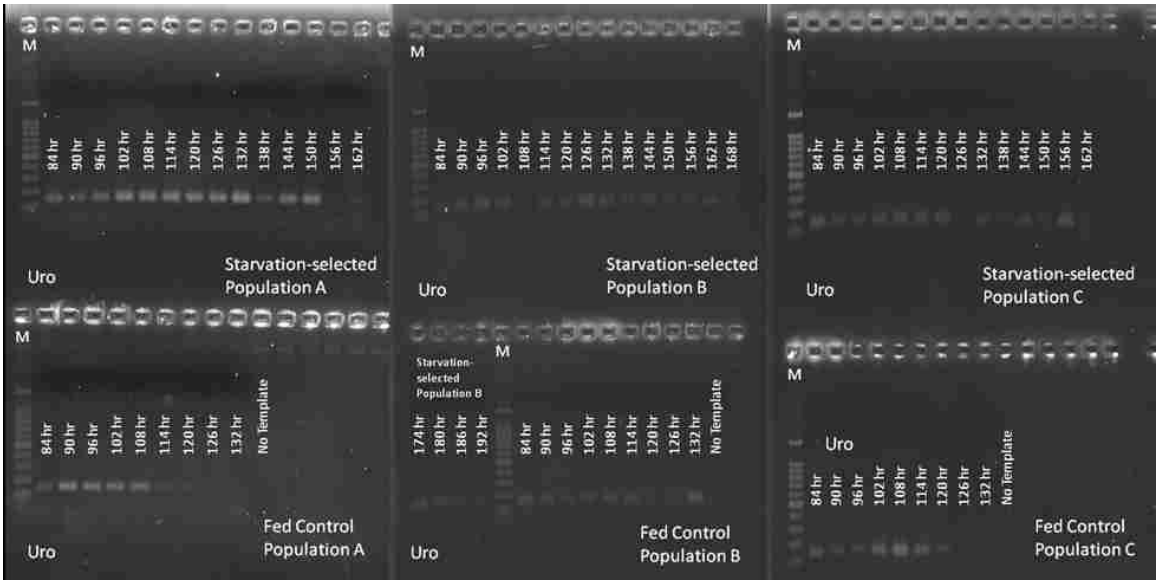
132 hr
No Template
Ddc
Fed Control Population C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Fed Control Population A
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Fed Control Population C

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Dissertation Title:

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