FOLLOWING THE FUEL: EXPLORING RESOURCE USE AND ALLOCATION DURING LIFE-HISTORY TRANSITIONS IN THE FLESH FLY, *SARCOPHAGA CRASSIPALPIS*

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010

To Erin, for your love and support

ACKNOWLEDGMENTS

This dissertation represents my work compiled as part of a group mentoring process with the ultimate goal of teaching me how to become a successful scientist. As such, a number of individuals have contributed their advice, technical expertise, constructive criticism and comments on a number of aspects of this research, and I thank you all. In particular, I would like to thank my doctoral committee, Mike Scharf, Peter Teal and Bruce MacFadden. I also thank my collaborator and mentor John Hatle for his guidance. Finally, and most importantly, I thank my major advisor Dan Hahn for his mentorship, his candor and his down to earth philosophy of science, which will continue to guide me throughout my career.

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December 2010

Chair: Daniel A. Hahn
Major: Entomology and Nematology

The acquisition and allocation of nutritional resources varies greatly based on an organism's life-history. Intermediary metabolism links the environment to the physiological response by governing the timing and magnitude of allocation to target tissues. However, our understanding of how allocation patterns change in response to the environment has been hindered by technical issues associated with tracking allocation. In recent years, these issues have been mitigated using stable isotopes of carbon and nitrogen to label resources and quantify allocation. Here stable isotopes are used to track resource use and allocation during major life-history transitions (i.e. reproduction and overwintering diapause) in the flesh fly, *Sarcophaga crassipalpis*.

Sarcophaga crassipalpis is a scavenging fly that feeds and larviposits on carrion, which is a temporally and spatially variable resource. These flies lay large clutches of eggs and although they can lay multiple clutches, the probability of mortality increases with time, therefore the quality of the first clutch is very important. Flesh flies are largely income breeders, relying mostly on adult acquired resources for reproduction. Adult resources are so important for reproduction that if only the minimal reproductive

threshold is met, flies will take nearly twice as long to provision their oocytes. This delay is likely an adaptation to the scavenging lifestyle of *S. crassipalpis*, allowing more time to locate additional resources. Resources acquired during the delay period were beneficial to reproductive timing and allotment, supporting this adaptive hypothesis. Additional findings suggest that the delay is not a physiological response to suboptimal nutrition and indicates that flesh flies have little capacity for plasticity in reproductive allocation.

Similar to reproductive allocation, during diapause flesh flies must budget resource allocation and metabolism to successfully survive overwintering. During diapause flesh flies rely primarily on lipids for fuel. Glycogen and glycerol levels also fluctuate, however, these compounds likely play a role in cryoprotection. However, during diapause break, flesh flies undergo a metabolic shift from lipids to a combination of lipids and carbohydrates. In summary, the results of this dissertation show that resource use and allocation decisions are tightly linked to the nutritional unpredictability of a scavenging lifestyle.

CHAPTER 1
INTRODUCTION

Insect Physiological Ecology

In recent decades, biology has become less specialized in nature, where once there were several distinct sub-disciplines within biology (e.g. ecology, physiology, cell biology, molecular biology, etc.) many with their own experimental and lingual quirks, now many scientists commonly integrate across a number of fields to investigate their particular question from a number of angles. The field of physiological ecology has grown as a result of this integrative trend, combining the study of organismal function with the interaction of the organism within an environment or within a population. The ultimate goal of physiological ecology is a comprehensive mechanistic view of how organisms interact with their environment (Chown and Nicholson 2004). Because of this integrative nature, studies within physiological ecology provide a unique perspective into the mechanistic processes that shape species distributions and evolution.

Insects in particular are an excellent model for understanding the link between physiology and ecology. Insects are a remarkably successful and diverse group of organisms; they inhabit every continent, and fill nearly every available niche. However, for any particular species of insect there are constraints on their ranges, most species inhabit ranges much smaller than the total size of the continent they live on (Chown and Nicolson 2004). There are a wide variety of biotic and abiotic effects that can constrain the range of a species; however, these likely vary within and between species throughout a particular range (Chown and Nicolson 2004). Teasing apart these complex interactions can be overwhelming. However, insects provide a variety of

interesting models for investigating how organisms respond to their biotic and abiotic environment.

Scavenging Insects: an Interesting Evolutionary Model

Insects occupy a number of ecological niches and have a wide variety of unusual and interesting life-history strategies. However, much of the physiological ecology literature has focused on the most common trophic niches, including primary, secondary, and tertiary consumers. While scavengers fit within this trophic scheme, they are not faced with the same biotic and abiotic challenges as most herbivores and predators. Herbivores and predators may face a certain amount of unpredictability in resource availability, although some of this unpredictability can be mediated by synchronizing life-history timing with the peak availability of resources. On the other hand, scavenging species, especially scavenging carnivores, have much greater unpredictability in resource availability and quality. In addition, when resources are available, intraspecific and interspecific competition is high because several vertebrate and invertebrate species are attracted to decomposing carrion. These characteristics make scavenging species interesting models to investigate life-history timing and the phenotypic consequences of variable nutrition.

The model organism examined in this dissertation was the flesh fly, *Sarcophaga crassipalpis*. This species has been an important model for stress physiology and overwintering diapause for a number of years (Denlinger 1972, Denlinger 1981). *Sarcophaga crassipalpis* has several characteristics that also make it a good model for evaluating resource allocation under variable environmental conditions. First of all, this species is relatively large; adults are approximately 1 to1.5 cm long and can weigh approximately 700 to 900 mg. This large size makes dissection, tissue isolation and

biochemical separation much easier than smaller species. Another valuable characteristic is their life-history; *S. crassipalpis* is anautogenous and therefore must locate suitable protein resources as adults to be able to provision its eggs. *Sarcophaga crassipalpis* provisions their eggs synchronously, producing 1 to 3 clutches during its lifetime. Once a clutch of eggs is fully mature, they are internally transported to a pseudo-uterus and hatch inside the mother. After a suitable larviposition site is located, she will lay active first instar larvae on the substrate. From the onset of oocyte development until larvae are ready to be laid takes between 8 and 12 days. Because decomposing carrion is an ephemeral resource, it is likely that mothers will not feed and larviposit on the same substrate. Reproduction in the flesh fly, a key life-history milestone, is centered around a great deal of unpredictability when relying on a spatially and temporally patchy resource. From an evolutionary perspective, this unpredictability makes the flesh fly an excellent model to study resource allocation under variable conditions.

Resource Allocation and Trade-offs

A variety of physiological systems have been explored in an ecological context, including but not limited to; thermoregulation, thermal limits, water balance, respiration, metabolism, and nutrition. Among these, nutritional ecology is of fundamental importance because few environmental factors have the same profound ability to shape life-history as the quality and availability of food. It is no coincidence that the availability and quality of nutritional resources can greatly affect major life-history milestones (i.e. reproduction, metamorphosis, diapause, etc.) for many species. There is little doubt that nutritional quality and availability have a large influence on fitness.

The allocation of resources within an organism represents the physiological mechanism linking foraging to life-history (Boggs 2009). Within any organism there are four major resource allocation pools; foraging, growth, reproduction and maintenance (Boggs 2009). In order to successfully grow and reproduce, an organism must efficiently allocate resources across these pools. However, resources are rarely in abundance and when they are, often individual dietary components are limiting, leading to trade-offs. This highlights the importance of not only resource quality but also the importance of resource congruency (Boggs 2009).

When resources are scarce or of low quality, allocation patterns will often shift and resources will be reallocated to other pools. A common example of such a trade-off is the relationship between reproduction and survival, whereby if resources are limited, many organisms will allocate more resources towards somatic maintenance than reproduction (Zera and Harshman 2001). The prioritization of allocation is essential to ensure survival until higher quality resources are located. While this may come at a cost by reducing an organism's lifetime reproductive effort, the cost is much greater if the organism does not successfully reproduce at all. This example represents the classic "Y model" of a nutritional trade-off, whereby the resources available to an individual are divided amongst reproduction and survival (van Noordwijk and de Jong 1986). When resources are limited, allocation to one trait (survival) occurs at the cost of allocation to the other (reproduction) (van Noordwijk and de Jong 1986). However, this example is most likely an oversimplification, reduced resources likely affect multiple aspects of an organism's life-history, resulting in several smaller trade-offs that are not easily observable.

Because life-histories are the product of allocation and allocation is a result of intermediary metabolism, understanding the flux of nutrients though an organism and how they are allocated opens up a key window into the mechanisms constraining or facilitating life-history evolution. Unfortunately, most life-history studies that investigate resource allocation rely on correlational data (e.g. mass, fecundity, etc.) despite some authors cautioning that correlation between traits is not necessarily a direct measure of allocation (Zera and Harshman 2001). However, validating nutrient allocation can be very difficult, highlighting the importance of marking nutritional components to follow the allocation of resources to tissues or classes of macromolecules. Over the years, a number of creative and innovative methods have been developed to accomplish this task, ranging from fluorescent dyes to radiotracers. However, ensuring that the marking method is non-invasive and does not alter the natural activity of the organism is of the utmost importance. Stable isotopes have been used for this purpose for many decades in studies of plant ecology and physiology. While their use in animal physiological ecology is relatively recent by comparison, their popularity is spreading (Gannes et al. 1997). Stable isotopes have several advantages over their radioactive counterparts, including the ease of use and increased safety, lack of decay, ability to be uniformly incorporated into a diet, and consistent labeling instead of pulse-chase are among a few of the advantages which have provided new methods for investigating complex metabolic interactions. Likewise, the use of stable isotopes in entomological studies has increased exponentially within the last decade. Stable isotopes have been used for a wide variety of applications, from studying ecological interactions to biochemical labeling and nutrient tracking (Hood-Nowotny and Knols 2007).

Stable Isotopes in Entomology

The traditional use of stable isotopes in animal ecology has been to evaluate trophic interactions and to reconstruct an organism's diet under natural conditions. The majority of entomological studies using stable isotopes have had a similar ecological focus. These studies generally rely on analysis of nitrogen 15, because the heavy isotope bioaccumulates with trophic level (Vanderklift and Ponsard 2003). However, determining the trophic dynamics of insect communities can be notoriously difficult and can be inhibited by obscure habitats, social interaction, and mixed diets. Bluthgen et al. (2003) faced a number of these challenges when trying to elucidate the role of ants in a rainforest food web. They hypothesized that canopy-dwelling species of ants occupied a lower trophic level (feeding on plants and nectar) than ground-dwelling species. The predictions of their hypothesis held for δ^{15}N levels, as canopy dwelling ants that fed solely on nectar had the lowest δ^{15}N levels (indicative of primary consumers), and primarily ground-dwelling and predaceous ants had the highest δ^{15}N levels (indicative of higher trophic consumers). Predictably, omnivorous species that fed on both nectar and animal prey had δ^{15}N levels between the two extremes. Interestingly, Bluthgen et al. (2003) noted that one dominant omnivorous ant species had notably lower δ^{15}N levels in mature forests than in open secondary vegetation, indicating a change in trophic position depending upon habitat. Tayasu et al. (1997) found similar trophic variation in δ^{15}N levels of 21 termite species that feed on different substrates in a forest community. They analyzed the δ^{15}N levels of each species, and found that wood-feeding species had lower δ^{15}N levels than soil-feeing species. Similarly, species that fed on a combination of substrates had δ^{15}N levels that were intermediate between wood and

soil specialists (Tayasu et al. 1997). These studies have demonstrated the value of stable isotopes in establishing the trophic position of species in complex ecological communities.

Stable isotopes also have been used to distinguish between multiple larval hosts of polyphagous agricultural pests, aiding the characterization of host race interactions in the field. The European corn borer, *Ostrina nubilalis*, is a pest with two distinct host races, one that feeds on corn (C_4 photosynthesis) and another that feeds on hop (C_3 photosynthesis) and mugwort (C_3). The interaction and mating of the adults of the two host races has important implications for pest management and insecticide resistance (Bontemps et al. 2004, Ponsard et al. 2004). The difference in $\delta^{13}C$ values between the hosts of the two races, due to differences in the affinity for ^{13}C between C_3 and C_4 carbon fixation enzymes, has been exploited to identify the host races of adults and track their interaction (Ponsard et al. 2004). Bontemps et al. (2004) determined that despite spatial overlap in host race distribution, host races of this species are probably not interbreeding and not contributing genetic variation that could reduce insecticide resistance. Similar methods have proven successful in other pest species, such as the polyphagous Lepidopteran pest *Helicoverpa zea* (Gould et al. 2002). However, the limitations of this technique were evident in the inability to distinguish between the various C_3 hosts of the tobacco budworm, *Heliothis virescens* (Abney et al. 2008). Despite these limitations, stable isotopes have been useful for distinguishing the host origin of some species. Indicating the potential for use in future studies of polyphagous pest species.

While notable advances have been made with stable isotopes in ecological research, stable isotopes also have been used in a variety of physiological applications. They have been used as tracers to follow the movement of resources within insects (Hood-Nowotny and Knols 2007). O'Brien et al. (2000) characterized the allocation of both larval and adult nutrients to reproduction in a hawkmoth (*Amphion floridensis*). They found that carbon from artificial nectar (labeled with sugar from C_3 and C_4 sources that were both distinct from the larval host plant) was increasingly incorporated into eggs as time progressed (O'Brien et al. 2000). These data indicated the increasing importance of adult resources to reproduction over time. O'Brien et al. (2002) expanded on these findings by determining that the carbon from adult-derived resources was incorporated into non-essential amino acids that were provisioned to eggs, whereas, essential amino acids were all derived from larval resources. The nectar-based diet of the adult moth is extremely protein limited; therefore, hawkmoths are limited in the amount of eggs that they can provision, based on the amount of essential amino acids acquired as larvae (O'Brien et al. 2002). This trend in allocation has also been explored in other butterfly species, displaying a variety of life-history strategies (O'Brien et al. 2005). The limitation of adult derived protein has been useful in describing interesting life-history adaptations such as fruit and pollen feeding in butterflies (Fischer et al 2004, O'Brien et al. 2005). O'Brien et al. (2005) discovered that pollen-feeding allowed the longwing butterfly, *Heliconius charitonia*, to sequester additional adult-acquired essential amino acids for reproductive allocation. Although the use of stable isotopes in physiological applications is still relatively new, they have the potential to add new

insight into traditional physiological problems, such as resource allocation and life-history energetics.

The work of O'Brien and others has shown the possibilities of tracking nutrient flow with stable isotopes (Karasov and Martinez del Rio 2007). Based on their data, O'Brien et al. (2000) were able to construct a flow model for carbon allocated to reproduction from larval and adult resource pools. Some of the unknown variables in this model were the loss of carbon during respiration and the source of that carbon. O'Brien et al. (2000) postulated that the majority of carbon lost in respiration was from adult resources, although this hypothesis has not been empirically evaluated. One future direction of stable isotope research is to couple isotope analysis with indirect calorimetry to estimate the source and amount of carbon lost during respiration. The feasibility of this method has been demonstrated in vertebrates such as hummingbirds and nectivorous bats (Carleton et al. 2006, Welch and Suarez 2007, Welch et al. 2008). This method can also be expanded to investigate the incorporation and metabolism of oxygen and hydrogen isotopes (^{18}O and ^{2}H) by providing organisms with labeled water ($H_2{}^{18}O$ and $^{2}H_2O$), an application that may be useful for determining the relative importance of resource storage versus metabolism during different times in an insect's life cycle (Bederman et al. 2006). The broad application of stable isotopes coupled with the ease of labeling and analysis suggests that their use in entomology will continue for years to come. In addition, integrating these techniques with other methods of observation will lead to new and exciting avenues of entomological research.

Aims and Scope

This dissertation focuses on using the emerging stable isotope methodologies to shed new light on our knowledge of resource use and allocation during nutritionally

stressful life-history transitions (such as reproduction and diapause) in insects. The central hypothesis of this dissertation is that resource use and allocation is flexible and can change to maximize organismal fitness (e.g. maximize reproduction and overwintering survivability) in variable environments. This central hypothesis is tested in five research chapters and can be subdivided into three functional sections that are targeted at three research aims:

Aim 1: Stable Isotope Chemistry and Metabolism

- **Chapter 2 hypothesis:** There is a concentration-dependent relationship between dietary concentration of ^{13}C and the discrimination against ^{13}C during metabolism (Caut et al. 2008).

Aim 2: Reproductive Allocation and Plasticity

- **Chapter 3 hypothesis:** This chapter is directed at characterizing the source of reproductive resources in *Sarcophaga crassipalpis* under ideal nutritional conditions (*ad libitum* feeding). I predict that the first clutch will contain substantial larvally-derived resources and that the contribution from capital will be reduced from the first clutch to subsequent clutches.

- **Chapter 4 hypothesis:** This chapter tests the hypothesis proposed by Hahn et al. (2008a) that a nutritionally induced delay in the timing of flesh fly reproduction is an adaptive response to allow flies more time to acquire additional resources to maximize the quality of the first clutch.

- **Chapter 5 hypothesis:** This chapter tests if reproductive allocation from capital and income resources changes as the timing of adult protein acquisition changes. We predict that reproductive allocation in flesh flies is flexible and that flies experiencing greater nutritional stress (i.e. adults denied protein longer) will allocate more capital stores towards reproduction (by sacrificing somatic resources) than flies that are not nutritionally stressed.

Aim 3: Diapause Metabolism and Substrate Use

- **Chapter 6 hypothesis**: This chapter tests the hypothesis proposed by Adedokun and Denlinger (1985) that pupal diapausing flesh flies rely on lipid stores to fuel the first half of diapause and once lipid reserves are depleted, flies switch to another unidentified metabolic substrate. In addition, I aim to identify the fuel that is used during the second half of diapuse.

CHAPTER 2
CARBON 13 DISCRIMINATION DURING LIPID BIOSYNTHESIS VARIES WITH DIETARY CONCENTRATION OF STABLE ISOTOPES: IMPLICATIONS FOR STABLE ISOTOPE ANALYSES

Introduction

In recent years naturally occurring stable isotopes have become an important experimental tool in animal physiological ecology, with isotopes of carbon (^{13}C) and nitrogen (^{15}N) the most commonly used (Martinez del Rio et al. 2009). Stable isotopes have been used for a wide variety of applications, from diet reconstruction and food web interactions (e.g. Ben-David et al. 1997, Barnes et al. 2007), to energetics and resource allocation (e.g. Welch et al. 2008, O'Brien et al. 2000). The utility of stable isotopes is based on the premise that the isotopic composition of an animal mimics the isotopic composition of its diet in a predictable manner (DeNiro and Epstein 1978, Gannes, et al. 1997). Naturally occurring or experimentally induced variation in the stable isotope concentration of dietary sources is a prerequisite for most ecological studies employing stable isotopes.

Natural variation in stable isotope concentrations can occur between organisms and even between different tissues within an organism (DeNiro and Epstein 1978). These differences are due to the routing and manufacture of organic molecules through biochemical pathways, which can lead to the specific distribution of isotopes within molecules (Rossman et al.1990). Routing refers to the direct incorporation of macromolecules from an animal's diet, although many macromolecules can also be biosynthesized within the animal from dietary components. During biosynthesis, isotopes can be fractionated by metabolic enzymes because of the increased molecular

bond strengths of compounds containing heavier isotopes relative to their lighter elemental counterparts (Martinez del Rio and Wolf 2005).

When animals consume multiple dietary sources that differ substantially in isotopic composition, the resource contributions from each source to the consumer's tissue can be distinguished. Untangling resource contributions to any particular tissue from a dietary component takes careful consideration and is typically accomplished using one of a variety of mixing models (Karasov and Martinez del Rio 2007). However, consumer tissues rarely match the dietary composition of their diet due to isotopic fractionation and metabolic routing (Martinez del Rio and Wolf 2005). The majority of mixing models incorporate an estimation of this difference in isotopic composition between the diet and the consumer, known as the discrimination factor ($\Delta^{13}C$). Discrimination factors can be measured directly in the lab, estimated mathematically, or inferred from the literature. However, one assumption often made when employing mixing models is that the discrimination factor for each dietary source is constant (Caut, et al. 2009). This assumption may be valid in some cases, however, some dietary sources may have considerable isotopic variation and a single discrimination factor may not always be suitable. Some authors have indicated that there may be a concentration-dependant relationship between discrimination factors and the diet (Caut et al. 2008, Henn and Chapela 2000, Hilderbrand et al. 1996, Ruess et al. 2005). If this is true, using a single discrimination factor for multiple dietary sources with substantial isotopic variation can lead to error in the estimation of source contribution (Caut et al. 2009).

In addition to isotopic differences between consumers and diet, there is also unequal discrimination across different classes of macromolecules (amino acids, lipids,

etc.). Carbon isotopic fractionation is particularly apparent in lipids, which are highly depleted in ^{13}C relative to proteins and carbohydrates. This fractionation occurs during the conversion of pyruvate to acetyl-CoA by the pyruvate dehydrogenase complex prior to fatty acid biosynthesis (DeNiro and Epstein 1977, Melzer and Schmidt 1987). Because consumers can vary in fat content, the low levels of ^{13}C in lipids can make the direct comparison of tissues from multiple consumers difficult. Depletion of ^{13}C in lipids has caused controversy in whether or not lipids need to be accounted for in stable isotope analyses involving ^{13}C, and both mathematical estimation and the chemical extraction of lipids from samples have been suggested as a solution for this problem (Post et al. 2007, Logan et al. 2008). However, these approaches are not appropriate in all situations because lipids themselves are often a resource pool of interest. Greater understanding of the relationships among diet, discrimination, and the biosynthesis of lipid is needed to improve the utility of mixing models in a wider variety of ecological systems

We tested the hypothesis that there is a concentration-dependant relationship between diet and ^{13}C fractionation, where diets rich in ^{13}C have greater discrimination by a consumer than diets that contain low concentrations of ^{13}C (Caut et al. 2008). Because lipids are highly depleted in ^{13}C we expected that concentration-dependant discrimination would be particularly apparent in this class of macromolecule. For this study, we investigated concentration-dependant discrimination in a simple controlled system using the bacterium *Bacillis subtilis* (Ehrenberg) Cohn. To determine if discrimination of lipids and other body components is dependent on the isotopic concentration of the diet, we investigated the relationship among whole tissue, lipid-

extracted tissue, and lipid fractions of bacteria raised on a nutritionally constant diet that varied only in ^{13}C concentration.

Materials and Methods

Culturing *B. subtilis*

We cultured the bacterium *B. subtilis* in minimal media ATCC broths containing only sucrose (98% of the carbon 5 g/l) and L-lysine ($\delta^{13}C$ = -11.76‰ 0.1 g/l) as carbon sources, forcing most bacterial components to be synthesized *de novo* (Atlas and Parks 1997). In addition, the ATTC broth contained a variety of salts, such as potassium phosphate (dibasic = 7 g/l, monobasic = 3 g/l), ammonium sulfate (1.5 g/l), magnesium sulfate (0.1 g/l), calcium chloride (0.01 g/l) and ferrous sulfate (0.005 g/l). Sucrose used in the broths was refined beet sugar (C_3 plant = low ^{13}C), cane sugar (C_4 plant = high^{13}C), or a mixture of the two, creating a linear gradient of five broth treatments with varying amounts of ^{13}C from low ($\delta^{13}C$ = -25.28‰) to high ($\delta^{13}C$ = -11.79‰) (Table 2-1).

Prior to inoculation of the experimental ATCC broths, a starter culture was prepared. The starter culture was prepared by combining *B. subtilis* spores in a tube containing 6 ml of Luria-Bertani (LB) Broth. The starter culture was incubated for 15 h at 37°C on an agitating shaker set at 150 rpm. Immediately following incubation, 0.3 ml of the starter culture was added to each 75 ml batch of experimental ATCC broth, which were kept at 37°C and agitated on a shaker set at 150 rpm. After 15 hours of incubation each 75 ml batch was centrifuged at 2500 rpm for 5 minutes to pellet the bacteria. At the time of experimental broth inoculation, cell density from the starter culture was $3.79 \times 10^6 \pm 1.07 \times 10^6$ and after 15 hours of incubation cell density was approximately two orders of magnitude larger $3.60 \times 10^8 \pm 2.11 \times 10^7$, therefore the starter culture

represented approximately 1% of the final sample after growth (paired t = 16.43, d.f. = 5, $p < 0.0001$) and contributed little if any to final ^{13}C values. Following centrifugation, the supernatant was discarded and the pellets were processed for either lipid extraction or whole tissue analysis.

Lipid Extraction

Bulk lipids from bacterial cells were extracted using a protocol modified from Folch et al. (1957). After centrifugation and separation from the remaining broth, bacterial pellets were re-suspended by vortexing in 5 ml of 2:1 chloroform: methanol and shaken at 200 rpm for 10 minutes. The samples were then centrifuged at 2500 rpm for 5 min, and the supernatant was drawn off and retained in a glass conical bottom vial. This extraction procedure was repeated two more times for each sample and the remaining tissue (i.e., the precipitate) was dried under nitrogen. Once dry, 600-700 μg of lipid-extracted tissue was placed into Costech 5x9 mm pressed tin capsules for stable isotope analysis.

The 2:1 chloroform: methanol fraction was phase partitioned by adding 1 ml of 0.9% NaCl in H_2O and vortexing for 30 seconds. Once the phases were partitioned, the organic phase was removed and dried under nitrogen. Immediately after drying, the remaining lipid was re-suspended in chloroform (DeNiro and Epstein 1978, Post et al. 2007). The lipid and chloroform mixture was dripped into Costech 5x9 mm pressed tin capsules containing approximately 2 mg of diatomaceous earth as a carbon-less substrate for solvent evaporation. The chloroform was subsequently evaporated under nitrogen. This process was repeated until approximately 700 to 800 μg of lipid had

been added to each capsule. The lipid-extracted and bulk lipid samples were replicated four times for each treatment.

Whole Bacterial Tissue Samples

Bacterial tissue pellets were lyophilized and approximately 600-700 µg of tissue was placed into Costech 5x9 mm pressed tin capsules for stable isotope analysis. Whole bacterial tissue samples were replicated four times per treatment.

Stable Isotope Analysis

The University of Florida Stable Isotope Geochemistry Lab processed all stable isotope samples analyzed in this study. Samples were first combusted in a Carlo Erba NA 1500 CNS elemental analyzer. The purified N_2 and CO_2 gas from the elemental analyzer was carried to a ConFlo II interface and then into a Finnigan-MAT 252 isotope ratio mass spectrometer. L-glutamic acid was used as a standard (NIST USGS40, standard $\delta^{13}C$ precision = 0.068 ± 0.006; n = 6).

The concentration of ^{13}C in a sample is expressed in delta notation. Delta notation represents a ratio of ^{13}C: ^{12}C in the sample as compared to the standard (Equation 2-1).

$$\delta^{13}C = (((^{13}C:\ ^{12}C \text{ in sample}/\ ^{13}C:\ ^{12}C \text{ in standard}) - 1) \times 1000 \qquad (2\text{-}1)$$

Calculation of Discrimination Factors

Discrimination factors are calculated as the difference between the isotopic composition of the diet and the consumer tissues ($\Delta_{diet} = \delta_{tissues} - \delta_{diet}$) (Martinez del Rio and Wolf 2005). The discrimination factor for each tissue fraction is estimated as the

difference between the $\delta^{13}C$ values of the carbon sources in the diet and the observed $\delta^{13}C$ values of the bacterial fraction.

Statistical Analyses

All statistical analyses were performed in JMP version 7.0.2 (SAS Institute, Cary, North Carolina, USA). The increase in $\delta^{13}C$ values across dietary treatment and sample classes were analyzed with linear regression. The effects of treatment and sample class on $\delta^{13}C$ were analyzed with ANCOVA. Means were separated using Tukey's HSD adjustment for multiple comparisons.

Results

Concentration-dependant discrimination of ^{13}C was present in all tissue fractions, compared to the diet (Figure 2-1) (Table 2-2). Discrimination between the diet and lipid ($\Delta^{13}C$) increased as the concentration of ^{13}C in the diet increased, from -2.72 ‰ at low ^{13}C concentrations to -15.5 ‰ at high concentrations (Figure 2-1). Similarly, concentration-dependant discrimination of the lipid-extracted tissue ($\Delta^{13}C$) ranged between -0.5 ‰ at low ^{13}C concentrations and -1.48 ‰ at high concentrations. Lipid was substantially more depleted in ^{13}C compared to other tissue classes (Table 2-2A). The slopes of both whole tissue and lipid-extracted tissue differed from the diet, showing discrimination particularly on the high ^{13}C diet. However, the slopes of whole tissues and lipid-extracted tissues did not differ from each other, likely due to the low lipid content of the bacteria (Table 2-2B).

Discussion

The variability of discrimination factors for ^{13}C and ^{15}N have been attributed to multiple factors such as diet quality, trophic position, omnivory, and metabolic routing

(Dalerum and Angerbjorn 2005, Hobson et al. 1996, Hobson and Clark 1992, McCutchan et al. 2003). Because of this, numerous authors have cautioned against using a single discrimination factor in isotopic mixing models (Caut et al. 2009, Dalerum and Angerbjorn 2005, Gannes et al. 1997). However, mechanistic explanations for the variability in isotopic discrimination have been lacking in the literature, despite the potential for this information to improve estimates of discrimination factors. DeNiro and Epstein (1977) determined the mechanistic basis for ^{13}C depletion in lipids due to the kinetic isotope effect during the conversion of pyruvate to acetyl-CoA by the pyruvate dehydrogenase complex. However, to our knowledge, our study is the first to carefully document the relationship between^{13}C discrimination during lipid biosynthesis and the ^{13}C concentration of diet.

There is a clear need for more studies investigating the dynamics of isotopic discrimination within single species across a range of diets. Hildebrand et al. (1996) noted that ^{13}C enrichment of bear plasma increased as ^{13}C in the diet decreased. In addition, Caut et al. (2008) demonstrated that discrimination factors were not consistent across diets of similar nutritional value in rats. They found a relationship between the dietary isotopic composition and ^{13}C discrimination in muscle, liver, and hair; where discrimination between the tissue and diet increase as dietary concentration of ^{13}C increases (Caut et al. 2008). However, they were unable to determine the cause of the discrimination. We hypothesized that this concentration-dependent relationship would be most apparent in the lipid fraction of the consumer due to discrimination by the pyruvate dehydrogenase complex during lipid biosynthesis. As expected, we observed slight concentration-dependant discrimination in whole tissue and lipid-extracted tissue,

however, the discrimination between the diet and the lipid fraction was much greater than other fractions (Figure 2-1). We expected the lipid-extracted tissue to be substantially less ^{13}C depleted than whole tissue, but we did not observe this, probably due to the low proportion of fatty acids in bacterial cells. The small quantities of fatty acids in prokaryotic cells (< 10%) may result in a small isotopic shift in the tissue after lipid extraction, although, lipid extraction has been shown to have a greater effect on animal tissues that contain larger neutral lipid stores (Madigan et al. 2003, Post et al. 2007).

The trophic enrichment of stable isotopes has been well documented for ^{15}N, while an isotopic shift of ^{13}C in higher-level consumers have typically been disregarded or assumed to be negligible (Martinez del Rio et al. 2009, McCutchan et al. 2003). In higher-level consumers, the enrichment of ^{13}C in consumer tissues has been shown to be relatively small (typically between + 0.3 ‰ and + 1.7 ‰) and it is also highly variable depending on tissue and taxonomic class (McCutchen et al. 2003, Caut et al. 2009). In contrast, the dynamics of ^{13}C have been investigated more thoroughly in primary consumers. Several authors have noted that decomposing saprotrophic fungi are more enriched in ^{13}C than symbiotic mycorrhizal fungi in ecological systems, and are arguably a better indicator of trophic level than $\delta^{15}N$ measurements in these species (Hobbie et al. 1999, Hobbie et al. 2001, Högberg et al. 1999, Kohzu et al. 1999). To further explore this phenomenon, Henn and Chapela (2000) evaluated the growth of basidiomycete fungi from different trophic levels on enriched and depleted ^{13}C substrates. They found unique species-specific fractionation patterns that did not explain the trophic pattern observed in the field, suggesting that these differences were due to the differential

uptake and routing of ^{13}C (Henn and Chapela 2000). However, only whole tissue samples were analyzed, making inferences about organismal-level effects of routing to specific nutrient pools (e.g. protein, carbohydrates, lipids, etc.) and structures (e.g. fungal chitin, reproductive sporocarps, etc.) difficult. Ruess et al. (2005) took the trophic transfer of ^{13}C from primary consumers one step further by tracking the allocation of fatty acids in a simplified fungal-based food web. They raised fungi on enriched and depleted ^{13}C diets, then raised nematodes on the fungi and raised collembolan springtails on both the nematodes and fungi. Similar to other studies, they noted general trends in the isotopic structure of fatty acids, for example, palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) were relatively deplete in ^{13}C (Abraham et al. 1998, Ruess et al. 2005). However, specific fatty acid $\delta^{13}C$ and concentration varied depending on organism, diet, trophic level and ^{13}C content; this variation across lipid classes prevented a strong resolution of predictive patterns (Ruess et al. 2005).

The overall goal of our study was to use bacteria as a model to understand isotopic discrimination in more complex biological systems, for that reason we focused on whole tissue and larger tissue fractions. We did not observe any enrichment in ^{13}C in bacteria; rather we observed that the bacteria were depleted in ^{13}C across all of the tissue classes we tested relative to the diet. Both whole tissue and lipid-extracted tissue showed a small but constant increase in discrimination as the dietary concentration of ^{13}C increased from low to high, -0.5 ‰ to -1.48 ‰ respectively. Similarly, in the lipid fraction we observed a large but constant increase in discrimination as the dietary concentration of ^{13}C increased from low to high, from -2.72 ‰ to -15.5 ‰ respectively.

An important question is why do we see such dramatic discrimination against ^{13}C in bacteria, but enrichment in higher classes?

We believe the difference is not due to taxonomy, because the pyruvate dehydrogenase complex in gram positive bacteria, such as *B. subtilis*, is structurally and functionally similar to the multi-enzyme complex in eukaryotic cells (Voet and Voet 2004). Rather, we attribute this difference to the method of carbon incorporation into the bacterial tissue. In most organisms, dietary resources can be used to synthesize macromolecules *de novo*, route macromolecules in the diet directly to tissues, or a combination of biosynthesis and metabolic routing. However, our experiment differs markedly from most isotopic ecology studies in that the minimal media broth used to culture *B. subtilis* virtually eliminated carbon incorporation due to metabolic routing because it offered few amino acid carbon skeletons for incorporation (except for those from lysine). Therefore, the majority of *B. subtilis* cellular components had to be synthesized de novo. We see large negative ^{13}C discrimination in bacteria forced to biosynthesize cellular lipids compared to the slight ^{13}C enrichment in organisms that are able to incorporate lipids directly from the diet. This suggests that isotopic incorporation of fatty acids due to metabolic routing may play a larger role than biosynthesis in many organisms. While interesting, these results also serve to caution those working on organisms that feed on carbohydrate-rich dietary resources, because lipid biosynthesis and therefore ^{13}C discrimination may be more prevalent in these species.

Stable isotopes provide a valuable tool for understanding a wide variety of processes that were previously inaccessible to ecologists. With the increased use of stable isotopes and mixing models in physiological ecology, the interpretation of data

collected in the field relies on the ability to accurately estimate discrimination factors (Caut et al. 2009, Gannes et al. 1997). The future of this technique will rely on laboratory studies that provide a better understanding of the mechanisms that affect stable isotopes post consumption and the development of predictive models that explain these processes.

Acknowledgements

The authors would like to thank Diane O'Brien and John Hatle for their constructive comments and suggestions on the development of this manuscript. We thank Drion Boucias for providing the *B. subtilis* colony and Ale and Jim Maruniak for their assistance in culturing the bacteria. Finally, we thank Jason Curtis for analyzing all of the stable isotope samples presented in this paper and for his advice and expertise on all things isotopic. Funding for this research was provided by NSF-IOS-641505.

Table 2-1. *Bacillus subtilis* experimental broth treatments.

Treatment	Broth Carbon Source	$\delta^{13}C$ (sugar + lysine) ± SE
1	100% cane sugar + lysine	-11.79 ± 0.045
2	75% cane, 25% beet sugar + lysine	-15.08 ± 0.506
3	50% cane, 50% beet sugar + lysine	-18.68 ± 0.139
4	75% beet, 25% cane sugar + lysine	-20.35 ± 0.012
5	100% beet sugar + lysine	-25.28 ± 0.070

Table 2-2. A) ANCOVA model for □13C profiles of B. subtilis diet and tissue fractions. B) Regression data for sampled tissue classes. Letters denote slopes that were significantly different in the ANCOVA model after Tukey's HSD correction for multiple comparisons.

A)

Model	DF	F	P
Whole Model	7	7053.66	< 0.001
Diet	1	20087.02	< 0.001
Tissue Type	3	7819.89	< 0.001
Tissue type x Diet	3	1942.97	< 0.001
Error	12		
Total	19		

B)

Tissue	Slope		Intercept	R^2	P
Diet	0.135	A	-25.29	0.999	< 0.001
Whole Tissue	0.126	B	-25.57	0.999	< 0.001
Lipid Extracted Tissue	0.126	B	-25.73	0.999	< 0.001
Lipid	0.007	C	-28.08	0.933	0.0076

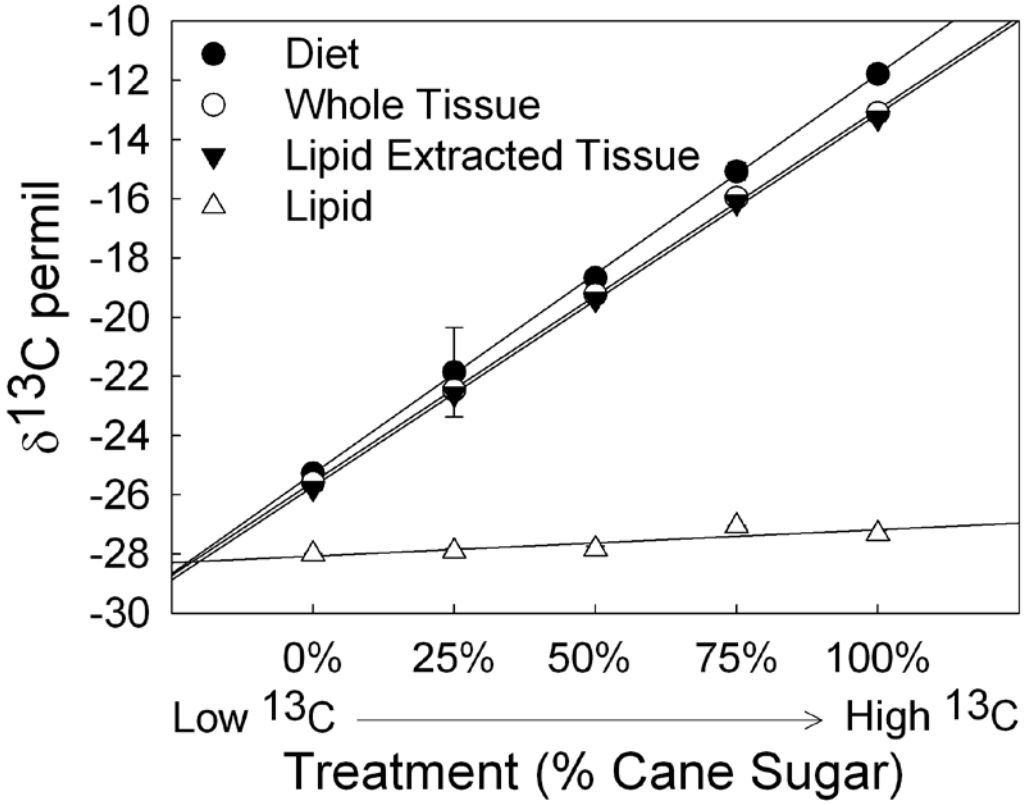

Figure 2-1. Relationship between isotopic values of diet, bacterial tissue, lipid-extracted tissue, and lipids. Symbols represent means and bars representing standard error are subsumed within the symbols.

CHAPTER 3
ALLOCATION FROM CAPITAL AND INCOME SOURCES TO REPRODUCTION SHIFT FROM FIRST TO SECOND CLUTCH IN THE FLESH FLY, *SARCOPHAGA CRASSIPALPIS*

Introduction

Understanding the role of nutrient acquisition, allocation and utilization to reproduction is fundamental to the study of life history evolution and physiological ecology (Stearns 1992). Reproduction requires substantial nutritional resources and represents a significant life history cost, by diverting resources away from other functions (Reznick 1985). The proportion of resources allocated to current reproduction as compared to the amount allocated to survival and future reproduction can influence individual performance and ultimately fitness (Boggs 1997a). Determining how nutrient allocation changes over an organism's lifespan, as the internal nutritional landscape changes, can improve our understanding of the mechanistic basis of allocation decisions in animals.

Resources used for reproduction can come from a variety of sources; however, these sources are often categorized into two general resource pools, capital and income (Jönsson 1997). Capital resources are acquired and stored prior to the reproductive period and are subsequently used to provision reproductive development, while income resources are acquired during the reproductive period and immediately allocated towards reproduction. Pure income breeding is arguably a more energetically favorable strategy because income breeders do not need to convert metabolic resources into storage molecules (e.g. triacylglycerides) and they do not need to maintain and carry large endogenous energy stores (Jönsson 1997, Jervis et al. 2005). Bonnet et al. (1998) argued that there are substantial costs associated with processing and

maintaining long-term energy stores in endotherms, but the low-activity lifestyles of ectotherms mitigate many of the costs associated with capital breeding and the benefits (reproductive flexibility) outweigh the costs. They predicted that capital breeding would be “far and away” the most common reproductive strategy in ectothermic vertebrates because of their low-energy lifestyle (Bonnet et al. 1998). Ectothermic insects, however, have a wide variety of energetic lifestyles ranging from nearly sessile (e.g. scale insects) to highly active (e.g. bees). Therefore, based on the predictions of Bonnett et al. (1998) we would expect to see the full range of breeding strategies from capital to income in these animals (Jervis et al. 2005).

Insects that eclose with all of their eggs fully provisioned are clearly pure capital breeders (Jervis et al. 2007, 2008). However, most insects provision at least some of their reproductive output from adult feeding. These insects have the potential to include both larvally-derived capital and adult-acquired income resources into their reproductive effort and most insects probably use a mixture of both resource pools (Jervis et al. 2007, 2008). Therefore, defining insects solely as capital or income breeders may be an oversimplification because this classification overlooks organisms that use a combination of capital and income resources during reproductive allocation. The majority of organism’s likely fall along a continuum between capital and income breeding, and their position along that continuum may change depending on external environmental conditions, internal body condition or over multiple bouts of reproduction (Jervis et al. 2007, Jönsson 1997, O’Brien et al. 2000, Stephens et al. 2009, Warner et al. 2008, Varpe et al. 2009). In addition, the predictable availability of resources may

influence where an organism falls along the capital and income continuum (Ellers et al. 2000).

Separating capital from income requires identifying precisely when the reproductive period begins, because this is when allocation from income begins (Stephens et al. 2009). Many holometabolous insects have a defined reproductive period which begins almost immediately following adult eclosion. Therefore, in these species pupation provides a convenient life-history transition that separates the pre- and post-reproductive period. Resources acquired as larvae and carried over into adulthood can be considered capital, whereas resources acquired by reproductive adults are considered income. Larval capital can be allocated towards growth or metamorphosis; it may be used during diapause, or stored as nutritional resources until the adult stage for reproduction or somatic maintenance (Sibley and Calow 1984). Similarly, adult income may be stored for later use or immediately allocated towards reproduction or somatic maintenance (Boggs 1997b).

A fundamental problem with identifying where organisms fall along the capital and income continuum is directly quantifying the source of the resources allocated to tissues of interest. In recent years, this problem has been mitigated with the use of naturally occurring stable isotopes as metabolic tracers (Warner et al. 2008). Using dietary components with different levels of carbon (^{13}C) and nitrogen (^{15}N) isotopes, capital and income resources can be specifically identified, allowing the direct quantification of resource allocation (Fischer et al. 2004, O'Brien et al. 2004). This technique was used to show the importance of income resources to reproduction in a presumed capital breeder, the hawkmoth *Amphion floridensis* (O'Brien et al. 2000, 2002). Even though

hawkmoths do not have access to substantial protein income as adults, O'Brien and colleagues (2000) found that the amount of income resources allocated to eggs increased over time, until approximately 50-60% of egg carbon was incorporated from adult nectar. In addition, they found that essential amino acids in the eggs were derived from larval capital; however, over time non-essential amino acids were increasingly synthesized from adult income nectar sources (O'Brien et al. 2002). Stable isotopes revealed the previously unknown importance of income carbon from nectar to reproduction in this species despite the presumed deficiency of the adult diet (O'Brien et al. 2000, 2002).

Unlike the hawkmoth, *A. floridensis*, the flesh fly *Sarcophaga crassipalpis* is able to feed on both protein and carbohydrate as an adult. Therefore, we expect the first clutch in the flesh fly to contain substantial larvally-derived capital and that the contribution from capital will be reduced from the first clutch to subsequent clutches in environments where resources are not limited. Hahn et al. (2008a) found that capital storage proteins (LSP-1) were depleted within a few days of adult eclosion whereas income-derived storage proteins (LSP-2) increased until the first clutch of eggs was provisioned in *S. crassipalpis*. However, protein storage represents only half of the reproductive allocation story because insect eggs contain roughly half protein and half lipid. In addition, neutral lipids are a common energy storage molecule in insects and most other animals because they provide more energy per weight than proteins. In this study, we use stable isotopes to evaluate the bulk allocation of capital and income resources within the flesh fly, *S. crassipalpis*. In addition, we use carbon stable isotopes to follow the allocation of neutral and polar lipids into somatic and reproductive

tissue across two reproductive clutches. We predict that we will see a large turnover from capital-derived lipids to income lipids in eggs between the first and second clutch, and we expect a similar pattern in somatic tissue as cellular components are replaced over time.

Materials and Methods

Insect Rearing

Experiments were conducted using a laboratory colony of *S. crassipalpis* maintained at the University of Florida following the methods of Denlinger (1972). Larvae were reared at a density of 80 individuals per 50 g of beef liver in a 25°C room with a 16:8 L:D light cycle. Liver was placed in aluminum foil packets that rested on a bed of vermiculite in a plastic shoebox (30 x 15 x 10 cm). After reaching the third instar, larvae wandered out of the foil packets and pupated in the vermiculite. After 5 days at 25°C, the pupae were sifted from the vermiculite and maintained in ventilated cups at 25°C until eclosion. On the day of eclosion, individuals were placed into screened cages (30 x 30 x 30 cm) and incubated at 25°C for the remainder of the experiment.

Experimental Diets

Stable isotopes are naturally-occurring isotopes of an element that have a different number of neutrons, and unlike radioactive isotopes, they do not decay. The most common isotopes used as dietary tracers are carbon (^{13}C) and nitrogen (^{15}N) (Hood-Nowotny and Knols, 2007). Stable isotopes are substantially less abundant than their natural counterparts, but their concentration can vary naturally in biological systems. Stable isotope concentration is typically reported as a ratio of the less abundant isotope to the more abundant isotope compared to a standard, commonly known as delta notation (expressed in $^{0}/_{00}$) (Equation 3-1). In the case of carbon,

animal and plant tissue is always more depleted in ^{13}C than the standard resulting in a negative number in delta notation; the less negative the number, the more ^{13}C the tissue contains.

$$\delta^{13}C = ((^{13}C: {}^{12}C \text{ in sample} / {}^{13}C: {}^{12}C \text{ in standard}) - 1) \times 1000 \quad (3\text{-}1)$$

C_4 plants have more ^{13}C ($\delta^{13}C$ ~ -13 ‰) than C_3 plants ($\delta^{13}C$ ~ -27 ‰) (Cerling and Harris 1999). In this experiment, we take advantage of this naturally occurring variation in isotope concentrations to create high and low ^{13}C labeled diets. We labeled the sugar source provided to the adult flies by using granulated sucrose from either sugar cane (C_4 plant, $\delta^{13}C$ = -11.26 ‰) or sugar beet (C_3 plant, $\delta^{13}C$ = -24.76 ‰). In addition, we provided both larval and adult flies with beef liver as a protein source; the liver used was from USDA organic grass-fed cattle raised in either Florida or Montana. In North America, the proportion of C_3 and C_4 grasses varies with latitude (MacFadden et al. 1999). C_3 grasses are more common at higher latitudes and C_4 grasses are more common in equatorial latitudes (Terri and Stowe, 1976). Therefore, the tissues of naturally grazing cattle raised at lower latitudes contain more ^{13}C (FL liver = $\delta^{13}C$ -17.3 ‰, latitude 29.17ºN) than those raised at higher latitudes (MT liver = $\delta^{13}C$ -25.7 ‰ latitude 45.22ºN). By providing larval or adult flies with either a combination of cane sugar and FL liver (high ^{13}C) or beet sugar and MT liver (low ^{13}C) we have created both high and low ^{13}C diets.

Larval flesh flies feed solely on the beef liver and the adults are provided liver, granulated sugar and water. The two isotopically-distinct diets were used to create four

experimental treatments, two of which were control treatments wherein the larvae and adults were fed the same diet (low: low – larvae: adults) and (high: high). In addition, two reciprocal switching treatments were set up, where the larvae and adults were switched from the high diet to the low diet and *vice versa*. Pupation was used as the switch point for the reciprocal treatments, because *S. crassipalpis* does not begin to provision eggs until after adult emergence (Hahn et al. 2008b).

Sample Preparation

For whole tissue analysis, insect tissue (soma or eggs) were frozen at -80 ºC then lyophilized. Next, the dried tissues were homogenized in a vibratory bead mill using zinc coated steel pellets. Approximately 700 µg of homogenized tissue was placed into Costech 5 x 9 mm pressed tin capsules for stable isotope analysis. Four replicates of each tissue (soma or eggs) were run for each of the four treatments immediately following eclosion, after the first clutch of eggs was provisioned and after the second clutch of eggs was provisioned.

Bulk lipids were extracted using a protocol modified from Folch et al. (1957). Insect tissue was mixed with 1.5 ml of 2:1 chloroform: methanol and homogenized in a vibratory bead mill using zinc coated steel pellets. Next the sample was shaken at 200 rpm for 10 minutes. The samples were then centrifuged at 2500 rpm for 5 min, and the supernatant was drawn off and retained in a glass conical bottom vial. This extraction procedure was repeated two more times for each sample. The 2:1 chloroform: methanol fraction was phase partitioned by adding 1 ml of 0.9% NaCl in H_2O and vortexing for 30 seconds. Once the phases were partitioned, the organic phase was removed and dried under nitrogen. Immediately after drying, the remaining lipid was re-

suspended in chloroform in preparation for fractionation of the neutral (storage lipids, i.e. triacylglycerols) and polar (membrane lipids) fractions.

The lipids were fractionated by loading the sample suspended in chloroform onto a 100 mg silica gel column. The sample was first washed with 10 ml of chloroform, one ml at a time, washing the neutral lipids off of the column. Next the sample was washed with 8 ml of methanol, washing the polar lipids from the column. Both the neutral and polar lipid fractions were removed, dried and resuspended in 300 µl of chloroform in preparation for stable isotope analysis. Both lipid fractions suspended in chloroform were dripped into Costech 5x9 mm pressed tin capsules containing approximately 2 mg of diatomaceous earth as a carbon-less substrate for solvent evaporation. The chloroform was subsequently evaporated under nitrogen. This process was repeated until approximately 700 to 800 µg of each lipid fraction had been added to each capsule. The lipid-extracted and bulk lipid samples were replicated four times for each treatment. Four replicates were run for each lipid fraction (neutral and polar lipids) for both tissue types (soma and eggs). Flies were sampled immediately following eclosion, after the first clutch of eggs was provisioned and after the second clutch of eggs was provisioned.

Stable Isotope Analysis

All stable isotope samples analyzed in this study were processed by the University of Florida Stable Isotope Geochemistry Lab. Samples were first combusted in a Carlo Erba NA 1500 CNS elemental analyzer. The purified N_2 and CO_2 gas from the elemental analyzer was carried to a ConFlo II interface and then into a Finnigan-

MAT 252 isotope ratio mass spectrometer. L-glutamic acid (NIST USGS40) was used as a standard.

Mixing Model

When using stable isotopes as resource tracers, it is important to account for the discrimination of the tracer as it is metabolized by the consumer. This discrimination results in differences in the composition of the consumer's tissue and the diet (Martinez del Rio and Wolf 2005). The easiest way to accomplish this is to rear individuals solely on both diets and measure the difference between the dietary ^{13}C concentration and the ^{13}C composition of the tissue of interest. To quantify discrimination, we included control treatments fed solely our high and low ^{13}C diets and sampled female somatic and egg tissue the same time as corresponding experimental treatments.

We were able to quantify the incorporation of carbon from the resource pulses into the eggs and somatic tissue using a simple two-source linear mixing model (Equation 3-2);

$$\delta^{13}C_{TISSUE} = (p \times \delta^{13}C_{INCOME} + d_I) + ((1- p) \times (\delta^{13}C_{CAPITAL} + d_C)) \quad (3\text{-}2)$$

where $\delta^{13}C_{TISSUE}$ is the consumer tissue of interest, p represents the proportion of the tissue derived from carbon from income. The term $\delta^{13}C_{CAPITAL}$ is the isotopic composition of the high diet and d_C represents the discrimination of the high diet by the consumer, $\delta^{13}C_{INCOME}$ refers to the isotopic composition of the low diet and d_I represents discrimination of the low diet by the consumer. All statistical analyses were performed using JMP version 7.0.2 (SAS Institute, Cary, North Carolina, USA) and mixing models

were calculated using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA).

Results

Sarcophaga crassipalpis is capable of laying multiple clutches, however, in this experiment substantial mortality occurred between the first and second clutches and almost no females from our starting pool survived past the second clutch. Over the two reproductive bouts we measured, flesh flies were nearly complete income breeders. However, as predicted the contribution of adult income was smaller in the first clutch (87.2 ± 1.2% income) than the second clutch (96.0 ± 0.9% income) (Figure 3-1A) (High: Low; t-test = 7.95; d.f. = 6; $p = 0.037$; Low: High; t-test = 173.61; d.f. = 6; $p < 0.0001$). Fly somatic tissue from both reciprocal treatments incorporated overall less carbon from income than eggs (Figure 3-1B). However, similar to the eggs, the amount of income carbon in the soma increased from the first clutch (43.2 ± 1.2% income) to the second (54.3 ± 1.8% income) for both treatments (High: Low; t-test = 10.42; d.f. = 7; $p = 0.018$; Low: High; t-test = 25.95; d.f. = 7; $p = 0.002$).

Egg lipids told a similar story as the whole tissue, that the neutral and polar egg lipids are primarily derived from adult income (Figure 3-2). Egg neutral lipids from both reciprocal treatments were not different from the corresponding control diets over both clutches. Lipids from the Low: High treatment did not differ from the High: High control, indicating that the egg lipids in the Low: High eggs came from the High ^{13}C adult diet. (filled points in Figure3- 2A). The same relationship was seen between the High: Low treatment and the Low: Low control (unfilled points in Figure 3-2A). Similarly, egg polar lipids did not differ from the corresponding controls over the two reproductive clutches, suggesting that polar lipids are also derived from adult income (filled and unfilled points

in Figure 3-2B). A Pearson's correlation analysis indicates that both polar and neutral egg lipids reflect a similar range of values (correlation = 0.908; count = 32; $p < 0.0001$).

The neutral and polar lipids from somatic tissues show a gradual turnover from capital to income as time progresses from newly eclosed females to females that have just provisioned their second clutch of eggs (Figure 3-3). Somatic neutral lipids from adult females from the Low: High diet treatment were initially different from those from the High: High diet control, because the flies had not yet been fed an income meal (filled points in Figure 3-3A). However, neutral somatic tissues from the first and second clutch did not differ from the High: High diet control, indicating that these adult lipids were increasingly derived from income resources (Figure 3-3A). A similar pattern occurred in neutral lipid turnover in the reciprocal High: Low diet treatment when compared to the Low: Low diet control (unfilled points in Figure 3-3A). Polar lipids from somatic tissue followed a similar pattern as the neutral lipid (Pearson's correlation = 0.726; count = 46; $p < 0.0001$). Somatic polar lipids from the High: Low diet treatment were different than those from the Low: Low control for the newly eclosed and first clutch individuals, but they were not different by the second clutch, indicating a shift from capital to income resources (unfilled points Figure 3-3B). The somatic polar lipids from the Low: High treatment approached the isotopic signature from the High: High control over time, however the signals were always distinguishable from one another (filled points Figure 3B).

Discussion

On the capital/ income breeding continuum, the flesh fly, *S. crassipalpis* falls solidly on the income breeding side. The flesh fly uses a small quantity of capital resources to provision the first clutch of eggs (10-15 %) and almost no capital in the

second clutch (2-5 %). This is interesting from an ecological perspective because of the hypothesized correlation between activity and reproductive tactics (Bonnet et al. 1998, Jervis et al. 2005). Bonnet et al. (1998) hypothesized that income breeding may be more prevalent in highly active species due to the cost of muscle maintenance and activity. *Sarcophaga crassipalpis* flesh flies are strong flyers, leading an energetically-intensive lifestyle, foraging widely for spatially and temporally patchy carrion resources and to find mates (Berrigan 1991). Perhaps there may be some advantage to maintaining less capital resources as a highly active forager (e.g. greater mobility, lower metabolic costs of storage, etc). Berrigan (1991), for example, found that female flesh flies, *Sarcophaga bullata* had reduced flight performance associated with an increase in mass during reproduction, and the greater mass associated with increased stores of larvally-derived capital may similarly hamper flight. Similarly, Warbrick-Smith et al. (2006) found evidence of a lipid storage cost in larvae of the moth *Plutella xylostella* by raising larvae on high- and low- carbohydrate diets. They found that over multiple generations, larvae raised on high-carbohydrate diets converted less carbohydrate to fat than individuals raised on a low-carbohydrate diet, suggesting that there is a fitness cost associated with excess fat storage. Jervis et al. (2005) investigated life history trade-offs associated with breeding strategies (i.e. ovigeny index) in lepidopterans. They evaluate the Lepidoptera because this group encompasses a wide range of flight activity levels ranging from completely apterous to highly mobile species. They hypothesized an association between breeding strategy and flight activity in a number of lepidopteran species, where individuals with high mobility are more likely to rely on income resources and those with lower mobility are more likely to utilize capital

resources for reproduction (Jervis et al., 2005). Several other insect groups have closely-related members with a wide range of activity levels (e.g. Hymenoptera, Coleoptera, Diptera, etc.) that would also make good candidates for reproductive allocation studies using stable isotopes.

Our finding that flesh flies are strong income breeders is supported by the egg lipid data confirming that polar and neutral egg lipids are derived from adult dietary components (Figure 3-2A and B). Because egg lipids are indistinguishable from adult income, the small amount of capital allocated to eggs is most likely in the protein fraction, because carbohydrates represent less than 2% of the total egg mass (Hahn, unpublished data). These results are similar to observations of reproductive provisioning in the continuously-laying *Drosophila melanogaster*. Eggs produced early in adult life are provisioned with a combination of larval and adult sucrose, however after approximately 10 days, the majority of sucrose carbon allocated to eggs came from adult income sources (Min et al. 2006). The hawkmoth *A. floridensis* follows a similar allocation pattern incorporating ever more income into daily egg production until income carbon incorporation to eggs stabilizes at approximately 50-60% adult income 10 to 15 days after adult emergence (O'Brien et al. 2000). Both of these species are similar to *S. crassipalpis* in that they are not carbon-limited as adults because all three species have ample dietary access to sugars. Most likely, the majority of lipids allocated to reproduction in *Drosophila* and hawkmoths is derived from adult income resources as well. However, both of these species continuously provision and trickle-lay their eggs over a period of time, whereas *S. crassipalpis* synchronously provisions large clutches of eggs simultaneously over several discrete reproductive bouts.

Income resources are not solely used for reproduction, some portion is used for somatic maintenance. The somatic whole-tissue data indicate that after the second clutch, approximately half of the carbon in the soma was derived from adult income. This is not surprising considering that flesh flies must maintain and replenish a variety of tissues including their large and energetically-expensive flight muscles (Berrigan 1991). Adult flies will not molt again, therefore, a considerable amount of capital carbon is tied up in the exoskeleton and wings as well as some internal tissues that were constructed solely with larval capital. In *S. crassipalpis*, the somatic neutral and polar lipids switch from a larval capital profile to a profile similar to adult income between emergence and the second clutch (Figures 3-3A and B). This turnover indicates that over time both neutral-stored lipids and polar lipids, largely representing cell membrane lipids, are replenished from adult income. These results too are consistent with data from other flies. *Drosophila* somatic carbon from sucrose is turned over from a larval to a partial adult profile over time, with a substantial larval signature maintained throughout adulthood (Min et al. 2006).

An interesting pattern can be seen in the high ^{13}C treatments in both egg and somatic lipids, where there appears to be a negative correlation between $\delta^{13}C$ and time (Figures 3-2 and 3-3). The depletion of ^{13}C in lipids is well known in isotopic ecology (DeNiro and Epstein 1977, Post et al. 2007). However, recent work suggests that lipid ^{13}C discrimination varies based upon the dietary concentration of ^{13}C (Wessels and Hahn 2010). Discrimination of ^{13}C in organisms forced to synthesize much of their lipids *de novo* is much greater than ^{13}C discrimination in organisms that can route lipids directly from their diet (Wessels and Hahn 2010). We hypothesize that the observed

correlation in the lipid fractions of the high^{13}C treatments is due to an increase in *de novo* lipid synthesis with time. However, further experimental tracking of dietary components into somatic and egg lipids will be needed to test this.

The relationship between capital and income allocation to reproduction under nutritional stress has not been investigated thoroughly in insects. In this study, flesh flies were fed protein and sugar *ad libitum*, whereas in the field, carrion and nectar are spatially and temporally patchy resources. The abundant availability of protein and lack of predation in laboratory conditions may have allowed the flies to allocate more income resources towards reproduction, with no nutritional pressure to force a trade-off between survival and reproduction. Perhaps we would have seen more capital incorporated if the flies were nutritionally stressed. Hahn et al. (2008a) demonstrated that restricting protein to adult female *S. crassipalpis* caused a delay in reproduction and a reduction in reproductive output, even though the minimal reproductive threshold had been met under these food-stressed conditions. Female flies receiving lower quality adult resources may have been mobilizing more capital towards reproduction. However, *S. crassipalpis* is anautogenous and does not maintain enough capital stores to reproduce without receiving a protein meal as an adult (Hahn et al., 2008a). This not true for all flesh flies, *S. argyrostoma,* for example, is capable of autogenous reproduction, showing that some species can either store excess capital reserves or that they may be able to reallocate internal resources towards reproduction in the absence of suitable income (Denlinger 1971).

The relationship between allocation strategies and resource availability has been investigated in other animal systems. For example, the multiple clutch laying tropical

snake, *Tropidonophis mairii* experiences seasonal variation in the abundance of resources throughout the breeding season (Brown and Shine 2002). These snakes allocate more capital towards reproduction during the wet (good resource quality) season and switch to income allocation during the dry (poor resource quality) season (Brown and Shine 2002). Similarly, Warner et al. (2008) observed that the multi-clutching lizard, *Amphibolurus muricatus*, use both capital and income derived resources to provision all of their clutches. Interestingly, they found that egg lipids were derived primarily from capital and proteins were derived equally from capital and income. They suggest that diet quality may influence the relative contribution of capital and income allocated to the eggs (Warner et al. 2008). While, studies linking reproductive allocation to environmental variation are not prevalent in the insect literature, a number of model organisms and novel reproductive strategies are ripe for investigation.

Our work establishes the base-line reproductive allocation patterns *S. crassipalpis* flies, common insect models for reproductive physiology and the physiology of stress resistance (Bylemans et al. 1994, Rinehart and Denlinger 2000). Understanding allocation profiles in a benign environment is a crucial baseline for future studies comparing how allocation strategies differ in stressful or variable environments (Boggs, 2009). Future work will focus on the effect of environmental variables on reproductive allocation, focusing on factors that may have shaped the evolution of reproductive strategies in this and other species.

Acknowledgements

We thank Kathy Milne for rearing and maintaining the laboratory colonies of *S. crassipalpis*. In addition, we acknowledge the invaluable assistance and expertise

contributed by Jason Curtis who ran our isotope samples in the Stable Isotope Geochemistry Lab at the University of Florida. This work was supported by NSF-IOS-641505 and the Florida State Agricultural Experiment Station. We also thank the HHMI Group Advantaged Training of Research Program for providing the funding and opportunity to involve Diana C. Jordan, an undergraduate scholar, in the planning and execution of this project.

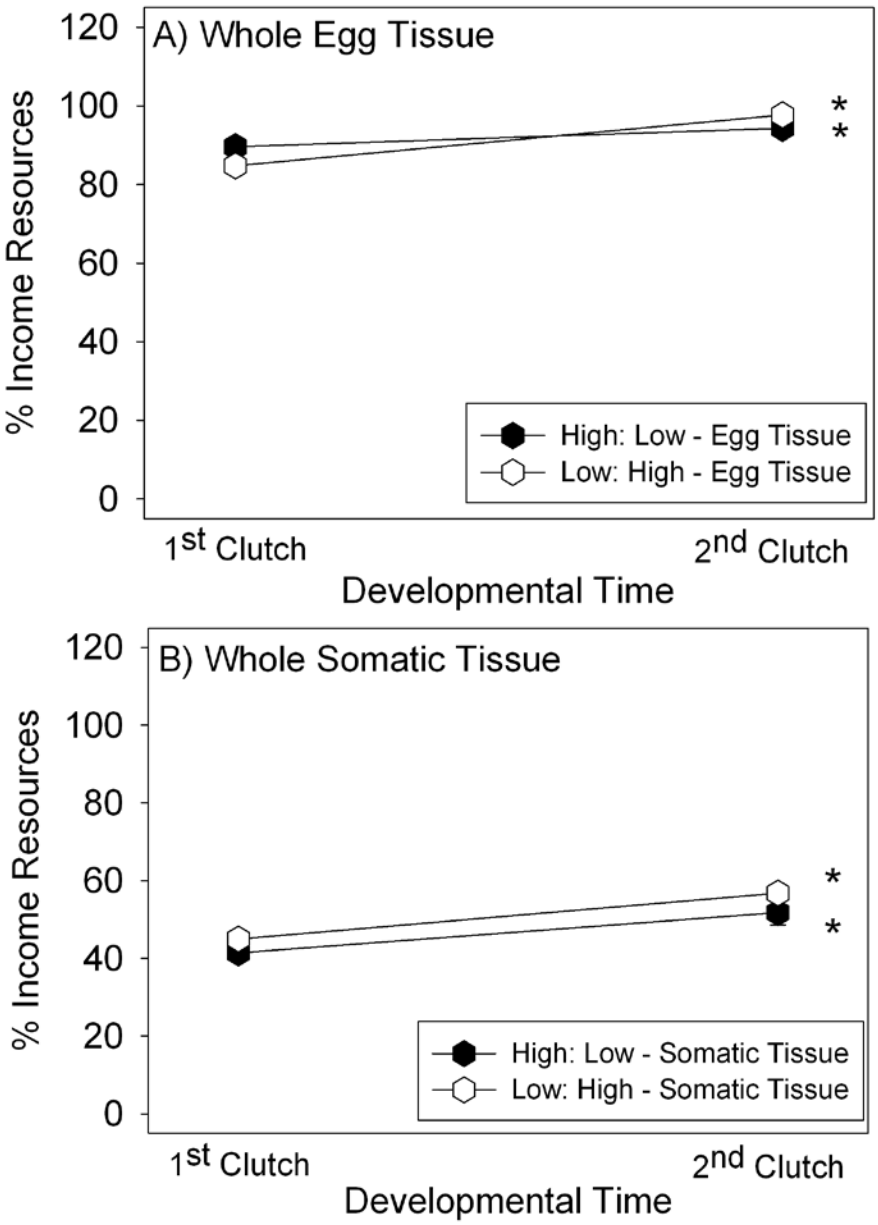

Figure 3-1. Flesh fly A) egg and B) somatic whole tissue contain less carbon from adult-acquired income sources at the first clutch compared to the second clutch. Treatments represent high and low ^{13}C diets switched from the larval to the adult stage (larval diet: adult diet) and error bars represent standard error (if error bars are not visible, they are subsumed within data points). Stars indicate a significance difference between the two developmental times within treatment (t-test, * = $p < 0.0001$).

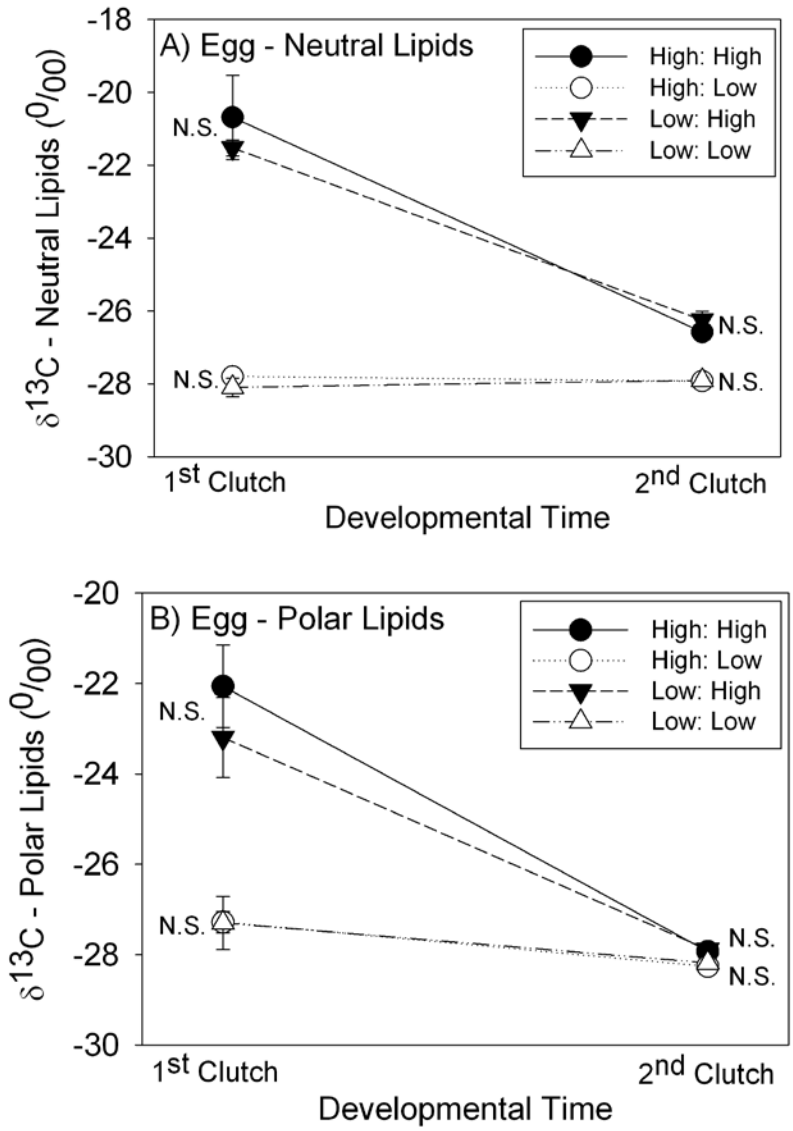

Figure 3-2. A) Neutral and B) polar lipids extracted from eggs across two reproductive clutches in the flesh fly. Treatments represent flies raised on high and low ^{13}C control diets and diets switched from the larval to the adult stage (larval diet: adult diet). Error bars represent standard error (if error bars are not visible, they are subsumed within data points). We used t-tests to determine differences between the switched diet and the corresponding control (filled circles vs. filled triangles and unfilled circles vs. unfilled triangles) within each clutch (N.S. indicates no statistical significance). We see that the control treatments are not different from the corresponding adult diets in the switched treatment, indicating that both the neutral and polar lipids in the eggs are derived from adult income.

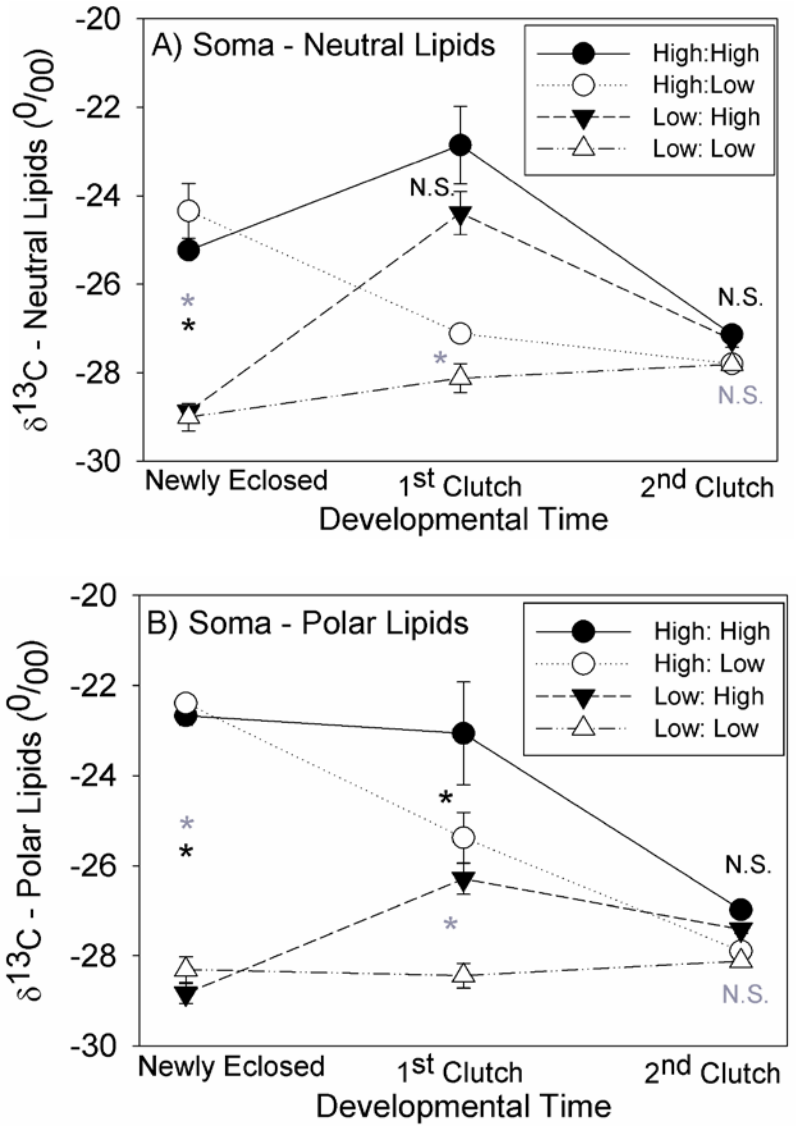

Figure 3-3. A) Neutral and B) polar lipid fractions extracted from flesh fly somatic tissue at three developmental time points as adults (immediately following eclosion, and after the first and second reproductive clutches). Treatments represent flies raised on high and low ^{13}C control diets and diets switched from the larval to the adult stage (larval diet: adult diet) and error bars represent standard error (if error bars are not visible, they are subsumed within data points). We used t-tests to determine differences between the switched diet and the corresponding control (filled circles vs. filled triangles and unfilled circles vs. unfilled triangles) within each developmental time point (N.S. indicates no statistical significance, grey * indicate significance between unfilled points and black * represent significance between filled points). We expected to see turnover in somatic lipid carbon from larval capital to adult income over time as new lipids were synthesized and incorporated into somatic tissue. As predicted, we see a shift in both neutral and polar lipids from larval capital to adult income over the three developmental time points.

CHAPTER 4
DOES IT PAY TO DELAY? BENEFITS OF DELAYING REPRODUCTION IN THE FLESH FLY *SARCOPHAGA CRASSIPALPIS*

Introduction

Much of an organism's life-history strategy is centered on preparing for and executing a few key life-history events (e.g. reproduction) that require a substantial quantity of resources. The efficient acquisition and allocation of nutritional resources has a profound influence on these processes (Boggs 2009). However, because resources are often limited, organisms must adjust life-history resource acquisition and allocation patterns to compensate for changing environmental conditions (Zera 2005; Boggs 2009). This plasticity is adaptive if it enables individuals to increase their fitness within a particular environment (Reznick 1990; Wingfield 2005; Shine & Brown 2008). The ubiquitous nature of life-history plasticity suggests that it is advantageous, allowing individuals to buffer changes in their environment and thereby efficiently allocate resources to growth, storage, and somatic maintenance (West-Eberhard 1989; Stearns 1992; Boggs 2009). Plasticity can be expressed at both the organismal (morphological and behavioral plasticity) and sub-organismal level (physiological plasticity) (West-Eberhard 1989; Hatle et al. 2004). While organismal plasticity is often more apparent than sub-organismal changes, both can have profound influences on life-history.

Reproduction is arguably the most important event in an organism's life-history, and as such it is an important component of fitness. For many organisms, the timing of reproduction and the magnitude of reproductive allotment are not static; they are flexible and highly tuned to environmental conditions (Bertness 1981; Moehrlin & Juliano 1998; Shine & Brown 2008; Wheeler 1996). Reproductive plasticity can be manifest in the timing of reproductive development, the magnitude of reproductive allotment, or a

combination of both. However, reproductive flexibility is not infinite, like any other trait there are limits to the extent of plasticity. The physiological control of life-history plasticity depends on numerous regulatory mechanisms (e.g. endocrine or genetic) each with their own feedback loops and constraints. These mechanisms can limit plasticity because they only allow for a certain number of physiological, behavioral, or anatomical states to occur simultaneously (Ricklefs & Wikelski 2002). Physiological limitations may restrict reproductive plasticity to relatively short windows of opportunity followed by fixed (or canalized) phases of development that are independent of environmental input (Hatle et al. 2000; 2001; 2004; Juliano et al. 2004; Twombly 1996). Determining whether reproductive plasticity is adaptive is important for ultimately understanding how selection may shape life-histories. Adaptive plasticity implies that an organism has the ability to compare its intrinsic condition with extrinsic environmental cues and to respond to these signals by altering its physiology; as opposed to non-adaptive plasticity that represents a phenotypic variation in response to being further away from an optimal environment (Reznick 1990; Juliano et al. 2004; Ghalambor et al. 2007). However, because not all examples of plasticity are adaptive, discerning adaptive responses from non-adaptive plasticity can be difficult and requires not only identifying plasticity, but also evaluating its influence on life-history traits (Reznick 1990, Ghalambor et al. 2007).

The majority of studies on reproductive plasticity in response to dietary input have been influenced by the classic model of amphibian metamorphosis proposed by Wilbur and Collins (1973), describing the development of frogs and salamanders in ephemeral ponds (Moehrlin & Juliano 1998; Hatle et al. 2004; Juliano et al. 2004). The

model predicts that individuals with access to high quality resources should delay metamorphosis to accumulate additional resources, while individuals experiencing poor resource conditions should undergo metamorphosis as soon as they reach the minimal nutritional threshold, allowing them to disperse to areas with greater resource abundance (Wilbur & Collins 1973). Clearly, resource availability will affect the time it takes to reach reproductive and developmental thresholds, so Wilber and Collins (1973) predicted that amphibians will show adaptive plasticity only after the minimum threshold has been reached. This concept of post-threshold plasticity can be extended to other major life-history events, such as reproduction. An important question is how reproduction will follow the predictions of this model; for example, will reproductive development in a poor environment result in faster post threshold reproductive development as predicted for metamorphosis?

Studies on reproductive plasticity in insects have not supported adaptive post-threshold plasticity as predicted by Wilbur & Collins (1973) (Boggs & Ross 1993; Moehrlin & Juliano 1998; Fischer & Fiedler 2001; Good & Tatar 2001; Bauerfeind & Fischer 2005). In contrast, most empirical studies with insects have shown that after achieving the minimal nutritional threshold for reproduction, allocation towards reproductive tissues is canalized (Juliano et al. 2004). Fixed allocation patterns suggest that insect reproductive plasticity is constrained by regulatory mechanisms that are activated once the minimal nutritional threshold has been met (Ricklefs & Wikelski 2002; Fronstin and Hatle 2008). While canalization may be common, it is not necessarily the rule. Hahn et al. (2008a) reported post-threshold plasticity in reproductive timing in the flesh fly, *Sarcophaga crassipalpis* Macquart. They found that adult female flesh flies

that were switched to a protein-restricted diet after reaching their nutritional threshold for reproduction took longer to reproduce and had lower reproductive allotment (smaller and fewer eggs) than those fed a high protein diet (Hahn et al. 2008a). This plasticity differs from the Wilbur & Collins (1973) model in that resource-poor individuals delay reproduction despite having already reached their minimum threshold, compared to resource-poor individuals that undergo metamorphosis rapidly. Perhaps adaptive plasticity or the lack thereof is best viewed from the evolutionary perspective of each particular species under investigation. Unlike larval amphibians that are constrained to an ephemeral aquatic habitat until metamorphosis, adult flesh flies are highly mobile income breeders that must acquire sufficient protein and carbohydrate resources to provision their eggs (Wessels et al. 2010a). In addition, flesh flies undergo energetically-demanding synchronous oocyte development and provision of large clutches of eggs, but they are short-lived as adults, suggesting that early reproduction is important. Considering the spatially and temporally patchy nature of their food sources (carrion and nectar), Hahn et al. (2008a) proposed that the delay in flesh fly reproduction might be adaptive if it allows flies more time to acquire additional resources to maximize the quality of the first clutch. While the concept of a reproductive delay could represent many things (e.g. time to mating, birth, reproductive development, etc.), we define the reproductive delay period as the time from the initiation of egg provisioning until a clutch contains fully mature, chorionated eggs.

Despite the importance of shifts in resource allocation to life-history plasticity, the flow of resources within an organism is one of the more difficult types of sub-organismal plasticity to measure directly (Zera & Harshman 2001). Evidence of this difficulty can be

seen in the large gap between theoretical and empirical studies of resource allocation in life-history strategies (Rivero & Casas 1999; Giron & Casas 2003). The difficulty of tracking internal resource allocation has been partly mitigated by labeling diets with both radioactive and naturally-occurring stable isotopes (Boggs 1997; O'Brien et al. 2000; Rivero et al. 2001). While both methods can be used to track allocation, stable isotopes have been used to both track and quantify resource allocation in multiple studies ranging from tracking the allocation patterns of larval and adult resources in Lepidoptera (O'Brien et al. 2000; 2002) to understanding the flexibility of reproductive allocation in lizards (Warner et al. 2008). In this study, we evaluate whether delaying reproduction can be beneficial to adult *S. crassipalpis*. We do this by providing isotopically-labeled resource "pulses" early, middle, and late during the delay period to determine if an additional resource pulse affects reproductive allocation or timing (Figure 4-1A). If the reproductive delay is beneficial and therefore consistent with an adaptive plastic response, we expect that resources acquired during the post-threshold delay period will result in faster development time, increased reproductive allocation, or a combination of both (Figure 4-1A). While we evaluate reproductive allocation using conventional organismal metrics (fecundity, egg size, etc.), we go beyond most other studies by quantifying the allocation of the pulsed resources to reproductive tissue using pulsed resources that have distinct stable carbon isotope profiles.

Materials and Methods

Animal Rearing and Experimental Design

Sarcophaga crassipalpis (flesh flies) used in this study were obtained from a laboratory colony maintained at the University of Florida according to procedures outlined in Hahn et al. (2008a) and Denlinger (1972). Newly-eclosed flies were hand-

sorted by sex into twelve screened cages (30 x 30 x 30 cm); each cage contained 150 females and 150 males. All cages were provided with water and granulated sugar *ad libitum* throughout the experiment. Of the twelve cages of adults, two were provided with liver from organic grass-fed cattle raised in Florida (a high ^{13}C food source, abbreviated FL liver) *ad libitum* throughout the experiment, two were provided FL liver for the first four days after eclosion, and the remaining eight cages were provided with FL liver for 2 days post-eclosion (Figure 4-1B). Of the eight cages in which adults were given FL liver for 2 days, two did not receive any additional resource pulses and the remaining six were given isotopically distinct resource pulses for an additional two days: on days 5 and 6, on days 7 and 8 , or on days 9 and 10 (Figure 4-1B). The flesh flies in this study are anautogenous and previous work has shown that the minimal quantity of protein required to reproduce is 3.21 mg per female; however, most females consume roughly 6 mg of liver prior to laying their first clutch (Hahn, unpublished data). In addition, females can acquire enough protein to provision a clutch of eggs from a single meal and they feed approximately once per day, therefore by providing *ad libitum* access to protein for two days we ensure that the flies have at least met their minimal nutritional threshold (Hahn et al. 2008). The isotopically distinct resource pulsing consisted of switching the granulated sugar source from cane sugar (high ^{13}C) to beet sugar (low ^{13}C) and providing *ad libitum* access to beef liver from organic, grass-fed cattle raised on a farm in Dillon, MT (low ^{13}C, abbreviated MT liver).

Female flesh flies do not begin to provision eggs until the fourth day post-eclosion (Hahn et al. 2008b). Therefore, starting three days post-eclosion, seven females were sampled from each cage daily until all of them contained fully-mature

eggs. The two cages provided with FL liver for 2 days were sampled for a total of 16 days, longer than in the other treatments because a portion of the females in this treatment did not fully provision eggs on any given sampling day. Females were frozen at -20 ºC immediately following sampling, and they were later dissected. Females were thawed on ice and dissected in ultrapure water, and their ovaries and any eggs in the uterus removed. Oogenic development was characterized by dissecting the ovaries and observing the state of the oocytes; oocytes were categorized using an eight-point scale ranging from previtellogenic follicules (1) to mature chorionated eggs (8) as described in Hahn et al. (2008a). Fully-mature eggs were removed from the uterus and counted as a measure of fecundity. The length and width of four mature eggs from each female were measured to the nearest 0.1 mm using a microscope-mounted ocular micrometer, and the mean value for each female was used in further analyses. The eggs from mature females and the remaining carcass (which we refer to as the soma) were stored in separate 1.5 ml microcentrifuge tubes and frozen at -80 ºC in preparation for stable isotope analysis.

Stable Isotope Analysis

In this study, we used stable isotopes of carbon (^{13}C – a common isotope for tracking diets) to track the incorporation of resources to reproductive and somatic tissue over time (Hood-Nowotny & Knols 2007). Stable isotope concentration is typically reported as a ratio of the less abundant isotope to the more abundant isotope compared to a standard, commonly known as delta notation (expressed in $^{0}/_{00}$) (Equation 4-1). In the case of carbon, animal and plant tissue is always more depleted in ^{13}C than the standard resulting in a negative number in delta notation; the less negative the number, the more ^{13}C the tissue contains.

$\delta^{13}C = ((^{13}C: {}^{12}C \text{ in sample} / {}^{13}C: {}^{12}C \text{ in standard}) - 1) \times 1000$ (4-1)

C_4 plants typically have more ^{13}C ($\delta^{13}C$ ~ -13 ‰) than C_3 plants ($\delta^{13}C$ ~ -27 ‰) (Cerling & Harris 1999). In this experiment, we take advantage of this naturally-occurring variation in isotope concentrations to create high and low ^{13}C labeled diets. We labeled the sugar source provided to the flies by using granulated sucrose from sugar cane (C_4 plant, $\delta^{13}C$ = -11.26 ‰) and sugar beet (C_3 plant, $\delta^{13}C$ = -24.76 ‰). In addition, we provided flies with beef liver as a protein source; the liver used was from USDA organic grass-fed cattle raised in either Florida or Montana. In North America, the proportion of C_3 and C_4 grasses varies with latitude (MacFadden et al. 1999). C_3 grasses are more common at higher latitudes and C_4 grasses are more common in equatorial latitudes (Terri & Stowe 1976). Therefore, the tissues of naturally-grazing cattle raised at lower latitudes will contain more ^{13}C (FL liver = $\delta^{13}C$ -17.3 ‰, latitude 29.17ºN) than those raised at higher latitudes (MT liver = $\delta^{13}C$ -25.7 ‰, latitude 45.22ºN). By providing flies with either cane sugar and FL liver (High ^{13}C) or beet sugar and MT liver (Low ^{13}C) we have created both high and low ^{13}C diets.

All flesh flies in this experiment were raised on the high ^{13}C diet as larvae and were initially provided with this diet as adults. The adult treatments that received a resource pulse during the reproductive delay were switched to the low ^{13}C diet during the pulse period (Figure 4-1B). This separation allowed us to track and quantify the incorporation of carbon from the resource pulses. Prior to isotope analysis, insect tissues were frozen at -80 ºC, then lyophilized. Next, the dried tissues were homogenized in a vibratory bead homogenizer using zinc-coated steel pellets. Between

650 and 750 µg of homogenized tissue from each individual was placed into a Costech 5 x 9 mm pressed tin capsules for stable isotope analysis at the University of Florida Stable Isotope Geochemistry Lab. Samples were first combusted in a Carlo Erba NA 1500 CNS elemental analyzer. The purified N_2 and CO_2 gas from the elemental analyzer was carried to a ConFlo II interface and then into a Finnigan-MAT 252 isotope ratio mass spectrometer. L-glutamic acid (NIST USGS40) was used as a standard, results were reported versus VPDB for $\delta^{13}C$.

Stable Isotope Incorporation – Mixing Model

It is important to account for the discrimination of the tracer as it is metabolized by the consumer. Discrimination results in differences in the composition of the consumer's tissue and the diet (Martinez del Rio and Wolf 2005). The easiest way to account for discrimination is to rear individuals solely on both diets and measure the difference between the dietary ^{13}C concentration and the ^{13}C composition of the tissue of interest (Table 4-1). To quantify discrimination, we raised a cohort of *S. crassipalpis* solely on our high ^{13}C diet, and another cohort solely on our low ^{13}C diet, and sampled four females when they had fully-mature eggs present in their uterus. The eggs were dissected and the egg and somatic tissue were prepared separately for stable isotope analysis (Table 4-1).

We quantified the incorporation of carbon from the resource pulses into the eggs and somatic tissue using a two-source linear mixing model (Equation 4-2):

$$\delta^{13}C_{TISSUE} = (p \times \delta^{13}C_{HIGH\ DIET} + d_{HD}) + ((1- p) \times (\delta^{13}C_{LOW\ DIET\ PULSE} + d_{LDP})) \qquad (4\text{-}2)$$

where $\delta^{13}C_{Tissue}$ is the consumer tissue of interest, and p represents the proportion of the tissue derived from carbon from the high diet. The term $\delta^{13}C$ High Diet is the isotopic composition of the high diet and d_{HD} represents the discrimination of the high diet by the consumer, $\delta^{13}C$ Low Diet Pulse refers to the isotopic composition of the low diet and d_{LDP} represents discrimination of the low diet pulse. All statistical analyses were performed using JMP version 7.0.2 (SAS Institute, Cary, North Carolina, USA) and mixed models were calculated using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA).

Results

The non-pulsed treatments showed a reproductive delay similar to that observed in Hahn et al. (2008a) (Figure 4-2A). Receiving a resource pulse substantially decreased the time required to fully provision eggs in all pulsed treatments relative to flies that did not receive an additional resource pulse. Egg development time in individuals given the earliest pulse on days 5 and 6 post-eclosion was not different from the 4 day non-pulsed treatment, although both took slightly longer to provision eggs than the *ad libitum* treatment (Figure 4-2B). Development time in the 7 and 8 and 9 and10 pulse treatments was slower than the *ad libitum* and 4-day, non-pulsed treatments. In addition, egg development time did not differ between individuals given a pulse on days 7 and 8 and individuals given a later pulse on days 9 and 10 (Figure 4-2B). Most important, development time in the 7 and 8 and 9 and 10 day pulse treatments was faster than the 2-day, non-pulsed treatment, suggesting that pulsed flies were able to beneficially use the pulsed resources to decrease the time required to provision eggs (Figure 4-2B). The majority of females fed liver for 2 days were able to

develop fully-mature eggs by 16 days; however, a small portion (36%) did not completely provision their oocytes (Figure 4-2A) (Table 4-2).

Resource availability and pulse timing had a substantial effect on reproductive allotment in *S. crassipalpis*. There was no difference in fecundity between the *ad libitum* and 4 day non-pulsed treatments, but the 2-day treatment had fewer eggs (Figure 4-3A). Individuals given an early pulse (days 5 and 6) produced more eggs than individuals receiving the intermediate pulse (days 7and 8) which, in turn, produced more eggs than females receiving the late pulse (days 9 and 10) (Figure 4-3A), indicating that resources acquired earlier during the delay period had a greater effect on fecundity than those acquired later. Feeding treatment similarly affected egg size (Figure 4-3B). In the pulsed treatments, individuals provided with the earliest pulse (days 5 and 6) had larger eggs than individuals that received the two later pulses (days 7 and 8, and 9 and 10) (Figure 4-3B).

Female flies were capable of incorporating resources from pulses during the delay period into eggs, and the timing of the resource pulse affected the magnitude of resource allocation. Females provided with the earlier resource pulses (days 5 and 6, and 7 and 8) incorporated more carbon from the pulse into their eggs than those provided a later pulse (days 9 and 10) (Figure 4-4A). Similarly, females provided the earliest two resource pulses (days 5 and 6, and 7 and 8) were able to incorporate more carbon from the pulse into their somatic tissue than those provided with the later pulse (days 9 and 10) (Figure 4-4B). Further, for all three pulsed treatments, the percentage of carbon allocated to reproductive tissue from the pulse was greater than the percentage of somatic tissue from the pulse.

Discussion

We expected that if the observed reproductive delay in flesh flies was adaptive, flies receiving additional resource pulses during the delay period should benefit from those resources by allocating them to reproduction. Flies that received additional resources during the delay period had more eggs, larger eggs, and reproduced faster than individuals that did not receive additional resources. Flies that received a resource pulse later in the delay period provisioned fewer eggs than those that received a resource pulse early in the delay. Therefore, resources acquired late during the delay period did not enhance reproduction as much as those acquired earlier, suggesting that flesh flies cannot delay reproduction indefinitely and that resources acquired earlier in the delay period have more value for the current reproductive bout than those acquired later. The observed benefits of resources acquired during the delay period are consistent with adaptive post-threshold reproductive plasticity (diamond symbols in Figures 4-1A and 4-3). In the field, it is likely that many flies do not survive long enough to produce a second clutch; although, an important question for future research is whether resources acquired late during the reproductive delay can be stored for future use by those that will produce a second clutch of eggs.

We further support the organismal metrics of reproductive output by quantifying allocation of the pulsed nutrients towards reproduction and soma using stable carbon isotopes. Flesh flies allocated carbon from all of the resource pulses towards reproduction, but the timing of the pulse affected both the magnitude of allocation and reproductive timing. Therefore, the environmental variable that initiated the plasticity (e.g. more food) was itself involved in the adaptive physiological change (allocation of nutrients from that food to the eggs) in response to that plasticity. In general, flies

receiving early pulses were able to use the nutrients from this food to a greater degree than flies receiving late pulses. Individuals that received the early and intermediate resource pulses allocated approximately 15% more carbon from the pulse to eggs than those that received the latest pulse (Figure 4-4A). Similarly, females that received a late pulse had somatic tissues that contained less carbon from the pulse than did females receiving an early pulse. This was despite the fact that all pulse groups required a similar time period from receiving the pulse until they fully provisioned their eggs. For example, the earliest pulse group received additional protein on day 5 and completed egg provisioning on day 9, while the latest pulse group received additional protein on day 9 and completed provisioning on day 12. Hence, there is a clear link between the environmental change (food availability) and the physiology of the flies (allocation of the meal to eggs and soma), and this physiological ability to incorporate nutrients changes with age.

A critical question underlying the presence of the reproductive delay in *S. crassipalpis* is the biological significance of this strategy. *Sarcophaga crassipalpis* females are capable of producing multiple clutches of offspring as adults. In the laboratory, they regularly lay 1-2 clutches of eggs, but conditions in the field are likely to be more stressful and opportunities for multiple clutches are probably more limited in nature than in the lab. Therefore, an obvious question is why females would delay reproduction to maximize the first clutch, when they could presumably lay a rapid but lower quality first clutch, and focus on acquiring higher quality resources for the second clutch. The delay strategy suggests that there are substantial benefits associated with maximizing allotment in the first clutch of eggs. Presumably, the benefits are

substantial enough to allocate additional time towards, even at the cost of future reproduction. Life-history theory predicts that early reproduction is more likely to be successful, and therefore more valuable, than later reproduction because the chance of mortality increases with age (Magnhagen 1990, 1991; Stearns 1992). Results from a parallel laboratory study suggest that flesh flies experience relatively high mortality after the first clutch (under laboratory conditions a clutch is ready to lay in 10 days). When reared under a 1:1 initial sex ratio, as in this study, female mortality is approximately 56% by day 20, the expected time of the second clutch under lab conditions, and 89% by day 30, the expected time of the third clutch, when fed continuous liver (Bastea L.I., Brix K.V., Hatle J.D., unpublished data). Therefore, even under very good laboratory conditions there is a high probability of mortality prior to laying the second clutch and the ability to produce a high-quality first clutch may be particularly important in field populations. Future work will focus on determining the specific benefits of the delay by quantifying the number and quality of offspring that go on to reproduce themselves from mothers that reproduce early in the delay period versus mothers reproducing late in the delay period relative to the risk of maternal mortality.

Due to the cryptic nature of resource allocation, the role of nutrition in shaping reproductive allocation strategies is not well understood (Boggs 2009). Moehrlin and Juliano (1998) manipulated the quantity and timing of food availability to the grasshopper *Romalea microptera (=guttata)*. Diets were switched from high to low quantity and vice versa. *Romalea microptera* was able to detect food quantity for a short window of time after adult molt and to respond to that input by altering the timing and magnitude of reproductive allocation accordingly (Moehrlin and Juliano 1998).

However, if food quantity was switched after the detection window, no changes in age at oviposition were observed (Moehrlin and Juliano 1998). In the case of *R. microptera*, it appears that reproduction shows plasticity in response to nutritional conditions immediately after adult molt. However, unlike flesh flies, there appears to be a time threshold, after which reproductive allocation is fixed and cannot be "rescued" by switching to high quantity resources. Similarly, the cockroach *Nauphoeta cinera* gradually matures oocytes following adult eclosion (Moore & Sharma 2005). However, in this species, mating induces an increase in juvenile hormone synthesis that results in rapid oocyte development (Moore & Moore 2001; Moore & Sharma 2005). There is a direct fitness cost to delaying mating, wherein females that delay mating past peak receptiveness have reduced fecundity and take longer to lay their first clutch (Moore & Moore 2001). Cockroaches that are starved following adult eclosion have lower oocyte volume and greater oocyte apoptosis (Barrett et al. 2008). Barrett and colleagues (2008) hypothesized that the oocytes are being resorbed to allocate additional resources towards somatic functions. Not surprisingly, reproductive allotment is decreased when nutritional resources are scarce. However, non-vitellogenic oocytes were also resorbed, suggesting that starvation may also negatively impact future reproduction (Barrett et al. 2008).

The mechanistic basis of plasticity and canalization has long been a subject of debate. One leading hypothesis suggests that the transition from plastic to canalized phases of development is due to attaining a nutritional threshold, and then releasing an endocrine signal that commits the animal to the major life-history transition (Bradshaw & Johnson 1995; Hatle et al. 2004). This can create constraints in life-history

development, such as canalized phases of development. Canalization during changing environmental conditions may be the result of physiological lags associated with the endocrine cascades that initiate life-history transitions (Nijhout 1994; Ricklefs & Wikelski 2002). For most organisms, these constraints on the physiological progression of life-history transitions may explain why there are limits on the number of life-history options available at any one time. In contrast to this typical pattern of attaining a threshold and then becoming fixed, *S. crassipalpis* reaches a threshold that allows completion of reproduction but retains plasticity after attaining the threshold.

To our knowledge, no other examples of nutritionally-induced adaptive reproductive delays have been documented in the literature. Perhaps this is due to the difficulty of identifying adaptive plasticity and establishing a cause for the plasticity. Simply finding a correlation between poor resources and delayed development or reproductive timing does not indicate an adaptive response. Adaptive reproductive delays have been noted in several mammalian systems (Wolff 1997; Agrell et al. 1998), but most of these are associated with either interspecific (Ylönen 1989; Norrdahl & Korpimäki 1995) or intraspecific competition (Huck 1982; Packer & Pusey 1983; Digby 1995; Woodroffe & MacDonald 1995). While it is easy to understand the adaptive significance of a delay strategy under these stressful conditions, the physiological effects of nutritional stress are often more cryptic and require creative approaches to elucidate them.

Adaptive life-history plasticity remains largely unknown from a biochemical perspective, and only a few model systems have demonstrated such mechanisms. One example is the wing-polymorphic cricket, *Gryllus firmus* (Scudder), that has distinct

morphs for dispersal and reproduction (Zera & Harshman 2001). The two morphs differ in their metabolic allocation strategies, wherein the dispersal morph allocates resources to flight muscle maintenance and the reproductive morph allocates the majority of resources to ovarian development in early adulthood (Zera et al. 1994; Zera & Zhao 2006). Zhao and Zera (2002) linked this trade-off between somatic maintenance and reproduction to a physiological difference in lipid biosynthesis. Another clear example of physiological mechanisms and adaptive plasticity of metamorphosis was found in the western spadefoot toad, *Spea hammondii* (Denver 1997). Denver (1997) linked the accelerated metamorphosis observed by Wilber and Collins (1973) to the elevation of the stress-induced corticotropin-releasing hormone that controls a suite of hormonal regulators of metamorphosis (Denver 1997). These systems represent valuable tools for characterizing phenotypic plasticity at the sub-organismal level, providing a foundation for studying mechanisms underlying the evolution of phenotypic plasticity.

Our findings demonstrate a clear link between the timing of resource availability and a physiological shift in resource allocation patterns that ultimately increases reproductive allotment and decreases the time required to reproduce. They go beyond those of most other studies of reproductive allocation by showing that flesh flies are able to incorporate additional nutrients acquired during the reproductive delay period and that there are benefits associated with receiving these resources. These results are consistent with adaptive post-threshold plasticity in reproductive timing and provide the foundation for investigating the mechanisms underlying plasticity and canalization in resource allocation.

Acknowledgements

We thank Jason Curtis for his insight and assistance in processing our stable isotope samples. We thank Mike Scharf his valuable comments on this manuscript. This work was supported by funds from NSF-IOS-641505 and the Florida State Agricultural Experiment Station.

Table 4-1. Quantification of 13C discrimination of egg and somatic tissue from female S. crassipalpis raised on high and low 13C artificial diets.

Diet	Tissue	Average $\delta^{13}C \pm SE$	n
High	Soma	-16.36 ± 0.13	4
Low	Soma	-24.90 ± 0.09	3
High	Eggs	-14.76 ± 0.18	4
Low	Eggs	-24.75 ± 0.25	4

Table 4-2. Multivariable general linear model for the effects of treatment and timing on egg development.

Egg Development	d.f.	F	p
Whole Model	17	86.65	< 0.0001
Treatment	5	60.50	< 0.0001
Age: Days Post-Eclosion	1	165.15	< 0.0001
Interaction: Trt x Age	5	22.87	< 0.0001
Cage Effects	6	8.21	< 0.0001
Error	901		
Total	918		

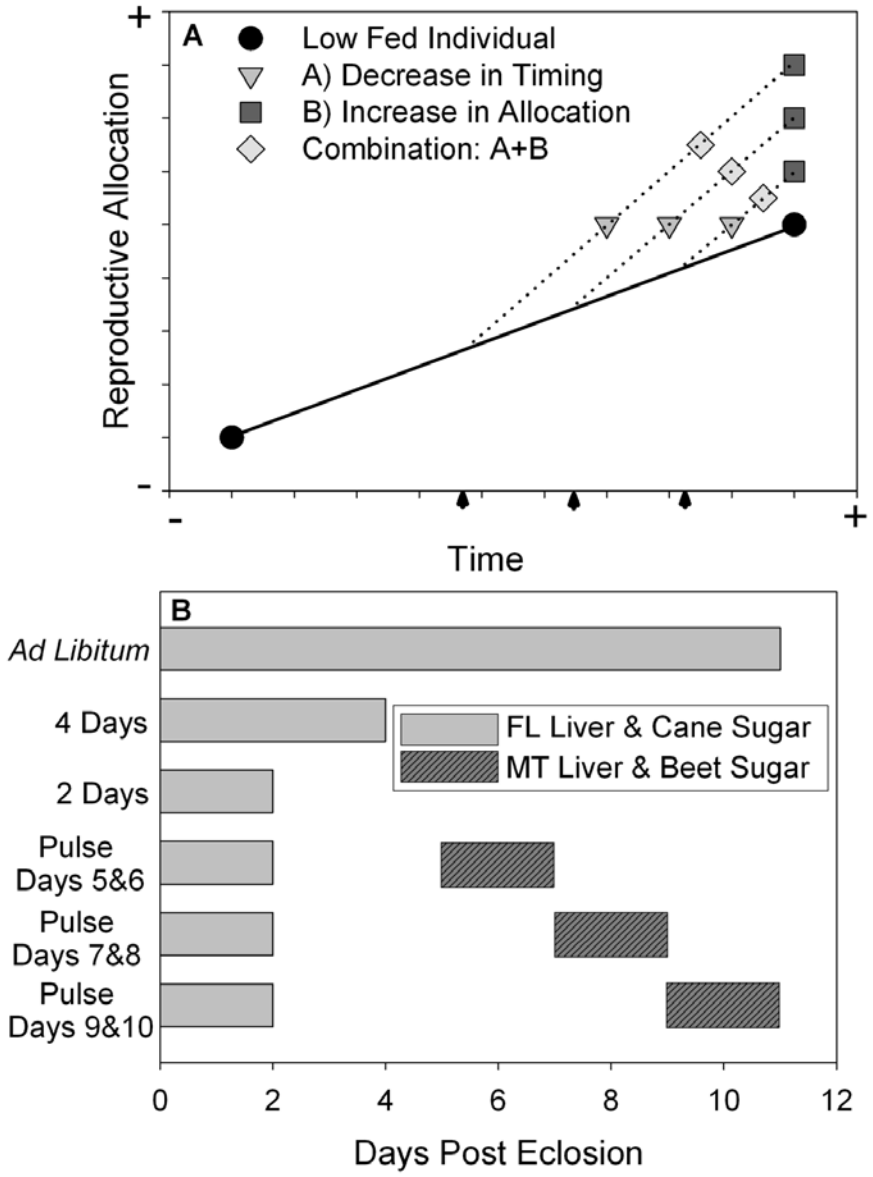

Figure 4-1. A) Predictive model for adaptive reproductive plasticity in *S. crassipalpis*. Arrows on the X-axis indicate three resource pulses at different times during oogenesis. The black circle symbols represent a theoretical allocation trajectory under low nutritional conditions (2 days of protein availability). The triangle symbols indicate what we would expect to see if the response to additional resource pulses only affected reproductive timing and not allocation. In contrast, the square symbols represent the response we would expect if additional resource pulses only affected reproductive allocation and not reproductive timing. Finally the diamond symbols represent the response we would expect to see if additional protein had an effect on both reproductive timing and allocation. B) Experimental design outlining the timing of resource pulses provided during the reproductive delay period.

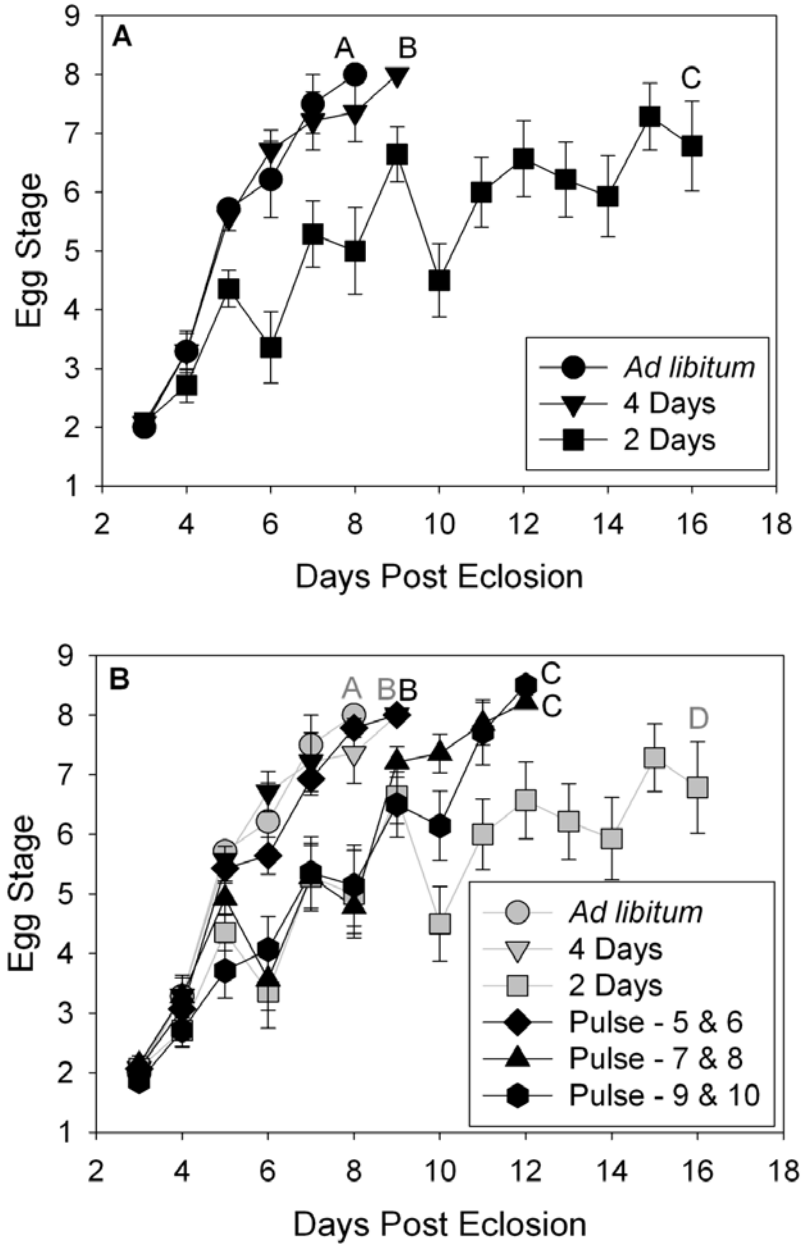

Figure 4-2. Egg development over time across feeding treatments, A) shows non-pulsed control treatments. The *ad libitum* and 4-day feeding treatments do not delay reproduction, while the 2-day feeding regime shows a delay in reproduction. B) Pulsed feeding treatments (black symbols) are overlaying the non-pulsed controls (grey symbols) showing the reduced reproductive timing when additional resource pulses are provided. Symbols represent means and bars represent standard error. Letters denote mean separation in a general linear model using Tukey's honestly significant difference.

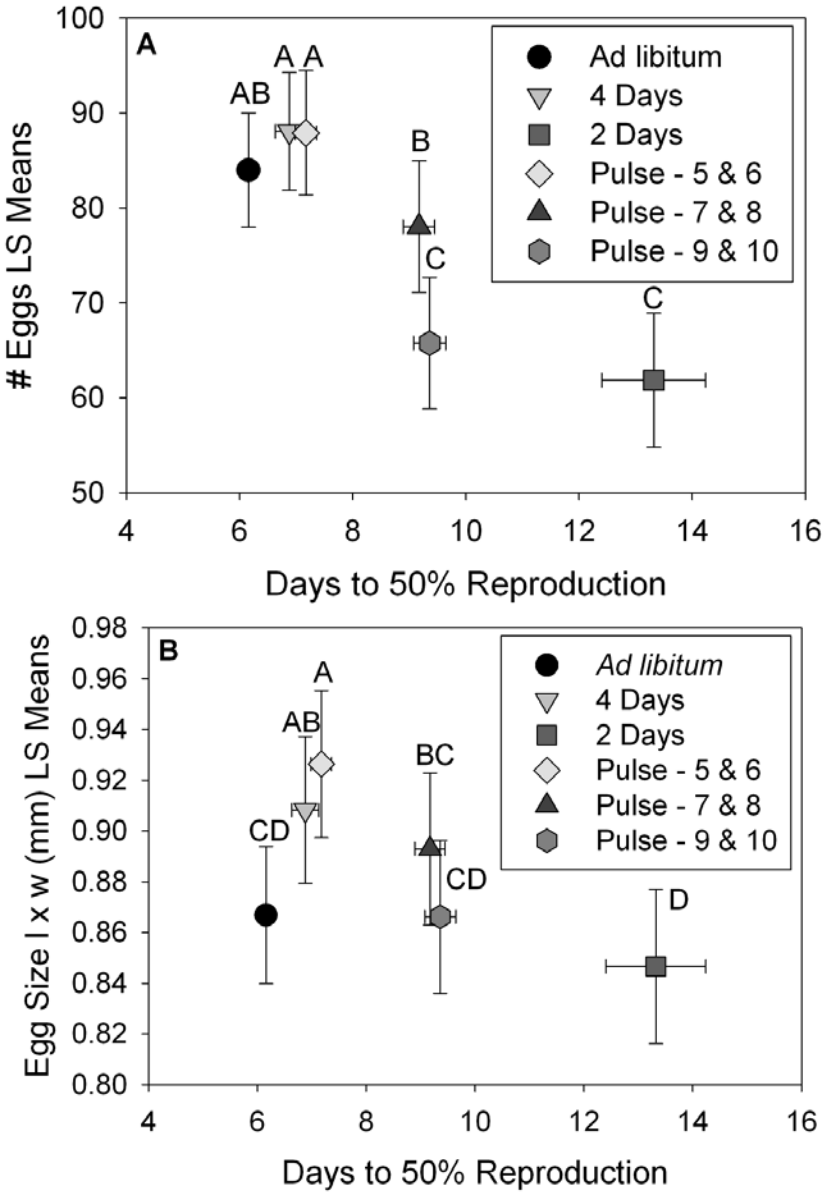

Figure 4-3. A) The relationship between reproductive timing (number of days until 50% of the population reached full reproduction) and fecundity and B) the relationship between timing and egg size (length x width). Both show the benefits of acquiring resources during the reproductive delay period, where individuals receiving earlier pulses have more eggs A) and larger eggs B) than those receiving later treatments. The symbols represent means and error bars represent standard error. Letters indicate separation of the mean egg size using a Student's t-test.

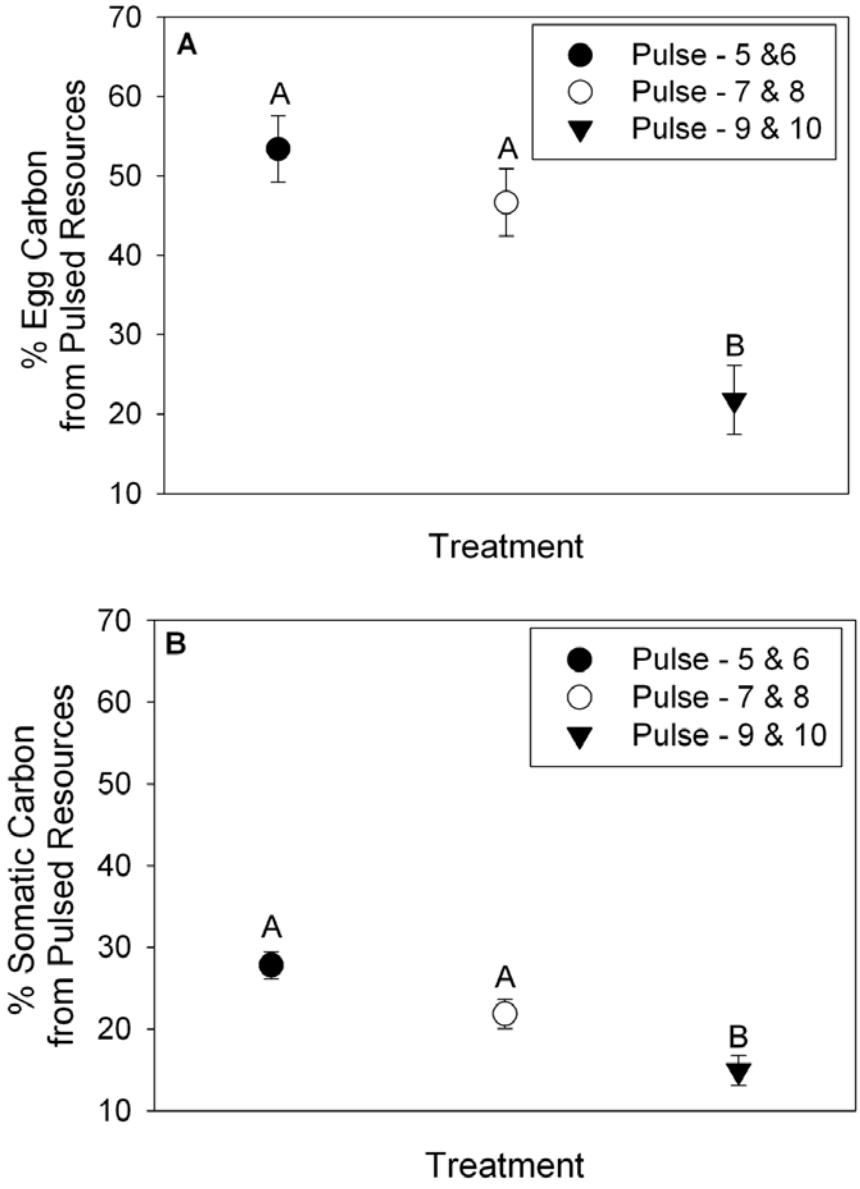

Figure 4-4. A) The amount of carbon (%) allocated to eggs from resources pulses provided during the reproductive delay and B) the amount of carbon (%) from pulsed resources allocated to somatic tissue. Both show greater allocation associated with early pulses although overall more resources were directed towards reproductive tissue. Bars represent means and error bars show standard error. Letters denote separation of the means using Tukey's honestly significant difference.

CHAPTER 5
RESOURCE AVAILABILITY AFFECTS REPRODUCTIVE ALLOTMENT AND TIMING, BUT NOT THE RATE OF OOCYTE DEVELOPMENT IN THE FLESH FLY, *SARCOPHAGA CRASSIPALPIS*.

Introduction

The acquisition and subsequent allocation of resources is critical to understanding an organism's life-history as well as for discerning larger patterns that underlie the evolution of life-histories (Boggs 2009). Not surprisingly, under natural conditions animals experience variation in both the acquisition of resources and in the quality of resources that are acquired. For example, some species may have a relatively consistent supply of food (i.e. generalist herbivores), while others may experience greater variation in the availability of resources (i.e. scavengers). Species that experience substantial variation in food availability or quality may frequently experience nutritional stress. This stress may force an organism into trade-offs where they preferentially allocate resources to one life-history trait at the cost of another (Zera and Harshman 2001). The presence of these trade-offs suggest that nutritional stress can have a profound influence on an organism's life-history.

While resource quality and quantity are undoubtedly important, the timing of resource acquisition also plays an important role influencing trade-offs (Zera and Harshman 2001). The timing of acquisition has been described most thoroughly in regard to reproductive storage or the lack thereof (Jönsson 1997). Organisms have been generally classified as capital or income breeders based on when reproductive resources are acquired. Capital resources are acquired and stored prior to the reproductive period and income resources are acquired during the reproductive period and allocated directly towards reproduction (Bonnet et al. 1998). Therefore, the time

that high quality resources are acquired holds different values for capital breeders than for income breeders. However, the majority of species likely do not fit perfectly within the definition of capital or income breeder, many species use a combination of stored and acquired resources for reproduction (Warner et al. 2008). In addition, the use of stored and acquired resources for reproduction can often change over time (O'Brien et al. 2000, Min et al. 2006, Wessels et al. 2010a). Previous investigation into reproductive allocation with the flesh fly, *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae), has shown that when offered continuous food from early in the reproductive maturation period they are primarily income breeders that use only between 10-15% capital stores to provision their first reproductive clutch (Wessels et al. 2010a). In addition, *S. crassipalpis* delays reproduction when provided only the minimal amount of protein necessary to reproduce, compared to flies that have full access to dietary protein. Previous work showed that even after females received enough nutrients to commit to fully provisioning a clutch of eggs, nutritional restriction later during reproductive maturation affected clutch dynamics (i.e. egg size and egg number). (Wessels et al. 2010b). The case has been made that this delay is adaptive, providing the scavenging fly time to locate additional protein-rich resources to maximize their reproductive output (Wessels et al. 2010b). However, an alternative explanation for the reproductive delay is that a trade-off is occurring and the flies are reallocating additional resources from other internal resource pools (e.g. flight muscle, fat body, etc.) towards reproduction. This strategy has precedent because other insect species have been known histolyze flight muscles to reallocate resources towards reproduction (Kaitala 1988, Zera et al. 1998). Insects have also been known to reduce reproductive

allocation by reabsorbing provisioned oocytes, presumably for allocation to other resource pools such as somatic tissue (Barrett et al. 2008).

Because reproduction is intimately tied to fitness, the determination of the optimal clutch size for an organism has been a subject of interest for evolutionary biologists (Brockelman 1975). There are physical limits on the maximum clutch size, as evidenced by the allometric relationship between ovary volume and body size in insects (Berrigan 1991). In several organisms, clutch dynamics (offspring number and size) have been reported to change under variable environmental conditions (Godfray et al. 1991, Hutchings 1991, Reznick and Yang 1993). However, a more difficult task is to understand how and why environmental conditions influence clutch dynamics. The first step to elucidating the mechanisms determining clutch dynamics is to understand plasticity in clutch size and timing under variable conditions.

In this study, we evaluate the extent of reproductive plasticity in the carnivorous scavenger *S. crassipalpis*. Scavenging species face unpredictable variation in resource availability, and must compensate reproduction accordingly. Therefore we evaluated reproduction in response to variation in the timing of the first adult protein meal in *S. crassipalpis* by restricting their access to protein as adults or by withholding protein for, 3, 6, 9, or 12 days. When protein is available, flesh flies can initiate oocyte development as early as 3 days post adult eclosion and depending on the amount of protein received, they can take up to 16 days to provision their first clutch of eggs (Wessels et al 2010b). Therefore, these treatments were designed to evaluate variation in adult protein acquisition early, midway, or late in the typical reproductive period. We test whether flesh flies are capable of mobilizing additional resources towards

reproduction in the absence of abundant adult acquired protein, using stable isotopes to determine if reproductive allocation from capital and income resources changes as the timing of protein acquisition changes with age. While *S. crassipalpis* can lay multiple clutches of eggs in the lab, in the field the probability of mortality increases with age so the success of the first reproductive clutch is very important. Because the first clutch is so important, we predicted that flesh flies that have experienced greater nutritional stress (i.e. adults denied protein longer) will allocate more capital stores towards reproduction (by sacrificing somatic resources) than flies that are not nutritionally stressed. We also evaluated the effects of protein starvation on the plasticity of clutch development by measuring the rate of oocyte development and the size and number of eggs.

Materials and Methods

Insect Rearing

Sarcophaga crassipalpis flesh flies used in this study were obtained from a laboratory colony maintained at the University of Florida according to procedures outlined in Hahn et al. (2008a) and Denlinger (1972). Larvae were raised on homogenized, organic, grass-fed ground beef from a farm in Missouri ($\delta^{13}C$ = -24.60; $\delta^{15}N$ = 4.40). Larvae were raised at a density of 80 individuals per 60 g of ground beef at 25ºC and exposed to a 16L: 8D photoperiod. Adult fly size can vary and individuals that are very large will likely have greater stores than those that are very small and therefore may have different allocation strategies in response to nutritional stress. To minimize the effects of body size variation, pupae were sorted by weight and individuals weighing between 100 and 110 mg were used for this experiment.

Experimental Design

Newly-eclosed flesh flies were sexed and hand sorted into five cages. The four experimental feeding cages contained 80 females and a fifth control cage contained 140 females receiving no protein as adults. All cages received water and granulated sucrose from sugar beets ($\delta^{13}C$ = -25.64; $\delta^{15}N$ = 0.00) *ad libitum* throughout the experiment. The four feeding cages were given a pulse of protein in the form of beef liver from corn-supplemented cattle ($\delta^{13}C$ = -15.41; $\delta^{15}N$ = 4.86) for 12 h on one of four days (day 3, day 6, day 9 or day 12). The beet sugar was removed from the cages 12 h before the protein pulse to allow time for flies to clear their crop and motivate feeding. Eight females were sampled from all feeding treatments for an eight day period beginning the day prior to the protein pulse. Six female flies were sampled from the no-protein control daily for days 3 through 19. All samples were frozen at -20ºC in preparation for dissection and stable isotope analysis.

Rate and Magnitude of Reproductive Allotment

Frozen samples were thawed and dissected in deionized water and depending on the progression of reproductive development, either the ovaries or the uterus were removed. The progression of oogenesis was recorded by staging the development of the eggs using an 8 point developmental scale, ranging from unprovisioned eggs (stage 1) to fully developed eggs in the uterus (stage 8), described in detail by Hahn et al. (2008a). Fully developed, stage 8 eggs, were counted as a measure of fecundity. In addition, the lengths and widths of four eggs from each individual were measured to the nearest 0.10 mm using a microscope-mounted ocular micrometer. Egg volume was estimated based on the shape of an ellipsoid as described by Rose et al. (1996).

Protein Allocation

We used stable isotopes to determine if the protein pulse provided to adult flies was allocated differently based on the timing of acquisition. Stable isotopes have become a common tool for evaluating nutrient allocation and there are several reviews that provide an excellent background on their use in biological systems (see Gannes et al. 1997, Hood-Nowotny & Knols 2007, Karasov & Martinez del Rio 2007). The diets used in this study were chosen based on their isotopic composition of ^{13}C. Both the larval diet and the adult sucrose have relatively low concentrations of ^{13}C ($\delta^{13}C$ = -25.60 and -25.64 respectively) (the more negative the $\delta^{13}C$ the less ^{13}C it contains) and the protein pulse has a greater concentration of ^{13}C ($\delta^{13}C$ = -15.41). The larval diet (MO ground beef) and the protein pulse (beef liver) do not differ appreciably in their ^{15}N values, and therefore ^{15}N values cannot be used to separate the diets, although it can be used as an indicator of nutritional stress (Gannes et al. 1997). The dietary sucrose does not contain any nitrogen.

We measured whole tissue ^{13}C and ^{15}N allocation to both egg and somatic tissue. Samples were first lyophilized and then homogenized in a vibratory bead mill. Approximately 600 to 800 µg of each dried and homogenized sample was weighed into a Costech 5x9 mm pressed tin capsule (Valencia, CA, USA) in preparation for stable isotope analysis. Because lipids can skew stable isotope analyses, especially in animals that have a carbohydrate-rich diet, we also extracted bulk protein from eggs for stable isotope analysis. To extract egg protein, tissue was placed into a microcentrifuge tube with 1.2 mL 20 mM Tris buffer (pH 9.0). The tissue was homogenized with an electric pestle and centrifuged at 14,000 rpm at 4ºC for 5 min. The supernatant was

drawn off and placed on a 1 cm tall column of diethylaminoethyl (DAEA) cellulose packed into a 5 cc syringe barrel. The cellulose was soaked in 20 mM Tris buffer (pH 9.0) overnight. After the sample was loaded on the column, an additional 6 mL of Tris buffer was washed over the column. Next, the column was washed with 7 mL of 1 M NaCl solution to elute the protein off of the column. The salt was removed from the protein fraction via dialysis. Spectra/ Por tubing, MWCO 12-14,000 kDa (Spectrum Labs, Rancho Dominguez, CA, USA) was placed over the top of centrifuge tubes that were placed into large beakers of water. Samples were dialyzed for 24 h during which the water was replaced every 8 h. The remaining fractions were mixed with 9 mL of cold acetone (-20ºC) and centrifuged at 7500 rpm (-4ºC) for 15 min then the supernatant was decanted off and the remaining protein pellet was dried under nitrogen. Approximately 600 to 800 µg of the remaining pellet was weighed into a Costech 5x9 mm pressed tin capsule (Valencia, CA, USA) in preparation for stable isotope analysis.

Stable Isotope Analysis

Analysis of $\delta^{13}C$ and $\delta^{15}N$ for each sample was determined by mass spectrometry at the University of Florida Stable Isotope Geochemistry Lab. Samples were first combusted in a Carlo Erba NA 1500 CNS elemental analyzer (Milan, Italy). The purified N_2 and CO_2 gas from the elemental analyzer was carried to a ConFlo II interface (Bremen, Germany) and then into a Finnigan-MAT 252 isotope ratio mass spectrometer (Bremen, Germany). L-glutamic acid (NIST USGS40) was used as a standard. Results were reported versus VPDB for $\delta^{13}C$ and versus AIR for $\delta^{15}N$.

Results

Adult female flesh flies require dietary protein to reproduce, flies that were restricted from protein as adults were only able to partially provision their eggs (up to stage 4) (Figure 5-1A). Females in treatments that were provided protein as adults were able to successfully reproduce and the rate off egg development was similar between females provided protein on day 3, 6, 9, or 12 after adult eclosion (Table 5-1, Figure 5-1B). Flies denied protein longer (fed day 9) had more eggs than flies that received an earlier protein meal at 3 days after adult emergence (ANOVA, $F_{(3,64)} = 5.40$, $p = 0.0022$) (Figure 5-2). However, flies fed protein earlier (fed days 3 & 6) had larger eggs than those fed later (fed days 9 & 12) (ANOVA, $F_{(3,64)} = 11.41$, $p < 0.0001$) (Figure 5-3). Both egg length and width differed between flies fed protein early (days 3 & 6) and those fed late (days 9 & 12), suggesting that the change in egg size was consistent across treatments (Figure 5-3). Overall, there was no change in total reproductive allotment (egg volume x egg number), indicating that while total egg # may increase with protein starvation, and egg volume decreases with length of protein starvation, the total investment in eggs is not different (ANOVA, $F_{(3,63)} = 2.41$, $p = 0.075$) (Figure 5-4).

The beef liver protein pulse provided to adult flies had a different stable isotope profile than the beef muscle that the flies were fed as larvae. Therefore, any changes in isotope concentration of the egg or somatic tissue would indicate that different quantities of adult and larval acquired resources were allocated towards that tissue. There was no difference in carbon and nitrogen isotope profiles of whole somatic tissue between any of the treatments (ANOVA $\delta^{13}C$, $F_{(3,12)} = 1.63$, $p = 0.235$; ANOVA $\delta^{15}N$, $F_{(3,12)} = 1.08$, $p = 0.393$). In addition, there was no difference in the carbon isotope

profiles of whole egg tissue and the protein fraction of egg tissue indicating that there is no difference in somatic investment towards reproduction between any of the treatments (ANOVA Egg Tissue, $F_{(3,10)} = 0.86$, $p = 0.491$; ANOVA Egg Protein, $F_{(3,12)} = 0.60$, $p = 0.630$) ($\delta^{13}C$ in Figures 5-5 and 5-6). However, there was a difference between the nitrogen isotope profiles in the egg tissue and egg protein fraction in flies that were fed protein on day 3 and those fed later (ANOVA Egg Tissue, $F_{(3,11)} = 8.46$, $p = 0.0034$; ANOVA Egg Protein, $F_{(3,12)} = 5.37$, $p = 0.0142$) ($\delta^{15}N$ in Figures 5-5 and 5-6).

Discussion

In *S. crassipalpis*, an adult protein meal is a requirement for reproductive maturation. We found that delaying the timing of the meal did not affect the rate of oogenesis in the flesh fly. In the absence of sufficient protein, oocyte development was stalled at stage 4, just after nurse cells were produced in the oocyte. However, upon receiving a protein meal, flies resumed provisioning their eggs, completing provisioning approximately seven days after feeding (Figure 5-1B). Flies that never received a protein meal, did not progress beyond early oogenesis (stage 4) and they never fully provisioned a clutch. These findings are interesting when compared to results reported by Hahn et al. (2008a), when flesh flies were restricted access to food earlier in reproductive development. Hahn et al. (2008a) found that flies provided *ad libitum* access to protein for 6 days after adult eclosion were able to provision a clutch of eggs within 7 days, in contrast flies provided access to protein for only the first two days after eclosion took nearly 14 days to provision their eggs. Wessels et al. (2010b) evaluated if this reproductive delay might be adaptive in the context of the scavenging lifestyle of flesh flies, to provide more time to find additional protein sources. Flesh flies were able

to allocate protein acquired during the reproductive delay towards their eggs and this resulted in larger eggs and faster reproduction, results consistent with an adaptive response (Wessels et al. 2010b). The consistency of the rate of egg development in this study support these conclusions, showing that even under nutritional stress (protein restriction) *S. crassipalpis* flesh flies are able to provision eggs in seven days. Therefore, the delay in egg development previously reported in this species is likely not the result of a physiological response to nutritional stress (Hahn et al 2008a, Wessels et al. 2010b).

We found that there was no difference in carbon stable isotope profiles in the somatic tissue, egg tissue, and egg protein fraction between all of the treatments ($\delta^{13}C$ data in Figures 5-5 and 5-6). These findings are not in accord with our hypothesis that flesh flies would reallocate somatic resources towards reproduction under nutritional stress. These results suggest that *S. crassipalpis* has little plasticity in reproductive allocation. Perhaps under protein restriction, they are not capable of re-allocating resources from other storage pools (e.g. flight muscle, fat body, etc.) towards reproduction. In addition, whole somatic tissue and whole egg tissue had a much lower range of $\delta^{13}C$ values (-23.09 ± 0.04 and -23.33 ± 0.14 respectively) than the egg protein fraction (-19.14 ± 0.16) indicating that the somatic and whole egg tissues contain more ^{13}C. This difference is most likely due to the presence of fatty acids in the somatic and whole egg tissues, because fatty acids are known to be depleted in ^{13}C (Post et al. 2007, Wessels and Hahn 2010). There was no difference in nitrogen isotope profiles in somatic tissue between any of the treatments. However, nitrogen isotope profiles in the whole egg tissue of the day 3 fed treatment were higher than the 6, 9, and 12 day

treatments (δ^{15}N data in Figure 5-5). In addition, nitrogen isotope values in the egg protein fraction of the day 3 fed treatment were higher than the 6 and 9 day, but not the 12 day treatment (δ^{15}N data in Figure 5-6). The reason for the higher δ^{15}N values in the day 3 fed treatment (indicating more ^{15}N) is unclear. Nitrogen 15 levels have been known to increase with nutritional stress and with trophic level (Hobson et al. 1992, McCutchan et al. 2003). In this system, we see an increase in δ^{15}N due to trophic level because the original larval and adult diets have δ^{15}N values of 4.40 and 4.86 respectively and the flesh fly tissue is higher, averaging 9.85 ± 0.03 for somatic tissue, 8.60 ± 0.09 for egg tissue, and 8.96 ± 0.06 for egg protein. However, trophic position does not explain the difference in δ^{15}N values between the day 3 fed individuals and the treatments fed later. If the difference were due to nutritional stress, we would expect that flies denied protein longer would have greater δ^{15}N levels than those fed the earliest. An increase in δ^{15}N has been associated with complete metamorphosis in Diptera (Tibbets et al. 2008). Perhaps this phenomenon plays some role in the peak in egg δ^{15}N values in the early feeding treatment which occurred soon after the completion of metamorphosis. However, if this is true, the concentration of the ^{15}N rich resource pool must be depleted after eclosion (possibly through excretion of nitrogenous waste), otherwise we would expect that any increase in δ^{15}N due to metamorphosis would affect all egg treatments (Doi et al. 2007, Tibbets et al. 2008).

Our results show that flesh flies do not reallocate resources from somatic tissues towards reproduction, even when deprived of protein. While some insect species have been known to histolyze energy-expensive tissues, such as flight muscle, to reallocate those resources towards reproduction, we do not see this in *S. crassipalpis* over the

time scale of this study (Kaitala 1988, Zera et al. 1998). A trade-off between flight muscle and reproduction occurs in the cricket, *Gryllus firmus* (Orthoptera: Gryllidae) in response to population dynamics, where muscle allocation is greater in dense populations (favoring dispersal) and reproductive allocation is greater when population density is low (Zera et al. 1998, Zera 2005). An important question is why does the flesh fly not employ a similar strategy to try to provision at least one clutch when food is not readily available? In the field, *S. crassipalpis* is a scavenger that experiences a lot of unpredictability in food availability, whereas the generalist omnivore *G. firmus* probably does not experience the same variation in resource availability. In addition, because flesh flies feed and oviposit on carrion, resource availability and oviposition sites are intimately linked and both are spatially and temporally variable. Perhaps the necessity of locating additional resources and suitable oviposition sites explains why flesh flies do not sacrifice flight muscle or other somatic tissues for reproduction.

In our initial hypothesis, we predicted that reproductive allocation in *S. crassipalpis* would trade-off allocation in somatic tissues towards reproduction. Although this did not turn out to be the case, the presence of trade-offs are a common prediction in studies that compare life-history traits under environmental stress However, similar to our findings, trade-offs are often not detected in empirical studies. On the contrary, sometimes a positive correlation between life-history traits is observed. A classic example of this is the surprising results reported by Spitze (1991) on the water flea, *Daphnia pulex* (Leydig). In the presence of midge predators, water fleas grew faster, larger, and were more fecund than populations not experiencing predation (Spitze 1991). Raising the question of why natural selection would not favor the highly

fit phenotype in the absence of predatory stress (Reznick et al. 2000)? Often, these confusing results are observed in populations raised under laboratory conditions and become clearer when viewed in an environmental context. *Daphnia pulex* experiences predation pressure from several predatory species. However, some predators selectively prey on smaller individuals while others prefer larger individuals, because of this Spitze et al. (1991) hypothesized that natural populations of *D. pulex* likely do not experience constant predation pressure from any one predator. Therefore, the fast-growing phenotype would have higher fitness in the presence of some predators while the slow-growing phenotype would have higher fitness when exposed to other predators; therefore, selection maintains the phenotypic plasticity in the population (Spitze et al. 1991, Reznick et al. 2000).

While the presence of a positive correlation between life-history traits under stressful conditions is not an obvious prediction, the model developed by van Noordwijk and de Jong (1986) is the most widely-cited hypothesis explaining this phenomenon. The van Noordwijk and de Jong (1986) model predicts that if variation in resource acquisition is greater than the variation in allocation, then positive life-history correlations may appear. Although resource acquisition is relatively easy to measure, quantifying resource allocation is much more difficult. We have shown that it is feasible to track resource allocation in flesh flies using stable isotopes as metabolic tracers. In addition, we have shown that *S. crassipalpis* has little capacity for plasticity in reproductive allocation, even in the presence of large variation in protein availability. Because of these characteristics, this species might be a good model to empirically test the predictions of the van Noordwijk and de Jong (1986) model.

Acknowledgements

We thank Kathy Milne for her assistance rearing and maintaining the laboratory colonies of *S. crassipalpis*. We also thank the funding agencies that provided support to incorporate two undergraduate scholars in the development and execution of this study. The University of Florida Undergraduate Scholars Program provided financial support for Matthew Rourke and the HHMI Science for Life Program supported Ross Kristal. Additional support for this work was provided by NSF-IOS-641505 and the Florida State Agricultural Experiment Station.

Table 5-1. A) A multivariable general linear model for the rate of reproductive development. The model was further reduced twice by B) removing the unfed control treatment and C) further removing the outlying day 0 sampling points from the day 3 fed treatment.

A)

Rate of Oogenesis	d.f.	F	p
Whole Model	9	244.38	< 0.0001
Treatment	4	382.55	< 0.0001
Age: Day after Feeding	1	73.68	< 0.0001
Interaction: Trt x Age	4	143.55	< 0.0001
Error	320		
Total	329		

B)

Rate of Oogenesis	d.f.	F	p
Whole Model	7	142.48	< 0.0001
Treatment	3	5.61	= 0.001
Age: Day after Feeding	1	281.94	< 0.0001
Interaction: Trt x Age	3	1.878	= 0.1342
Error	224		
Total	231		

C)

Rate of Oogenesis	d.f.	F	p
Whole Model	7	123.93	< 0.0001
Treatment	3	2.24	0.0848
Age: Day after Feeding	1	127.85	< 0.0001
Interaction: Trt x Age	3	1.7304	0.1617
Error	216		
Total	223		

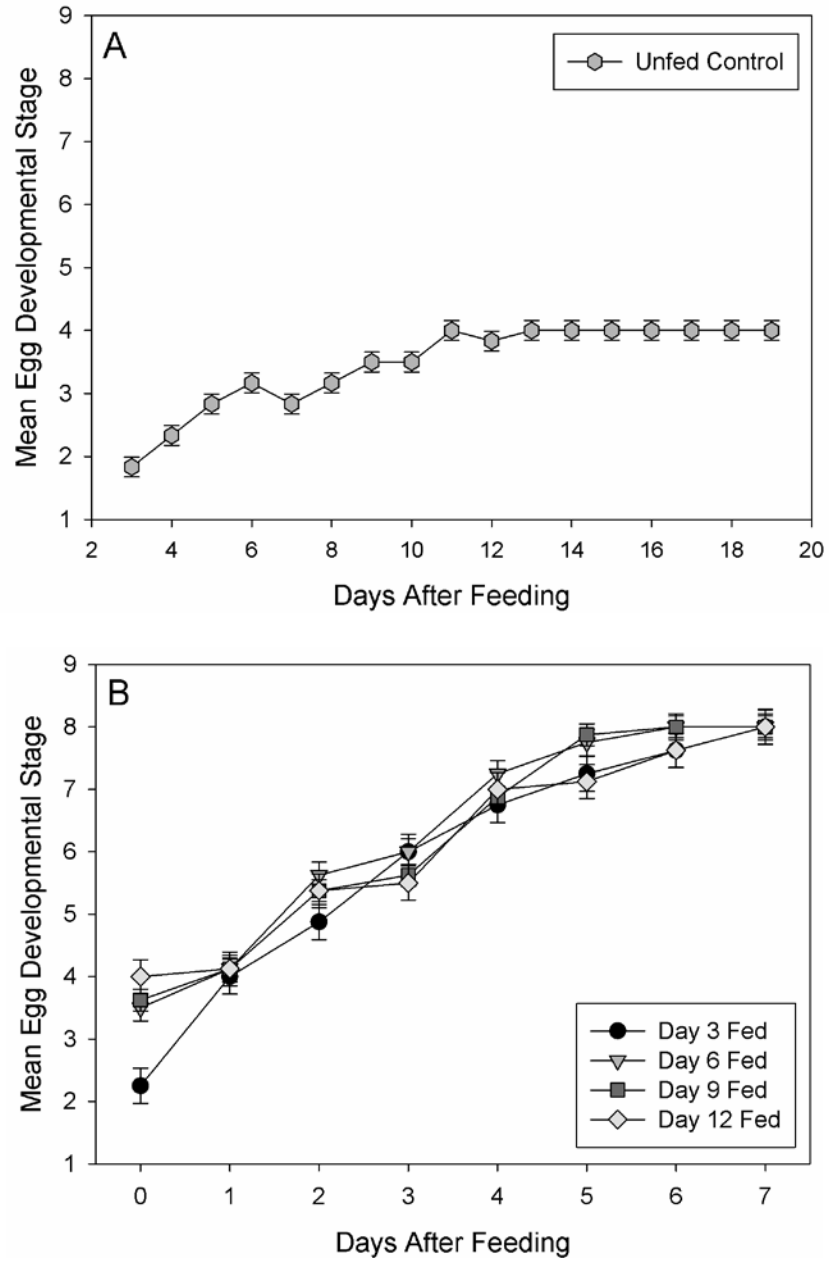

Figure 5-1. A) Incomplete egg development (fully developed eggs = stage 8) in females that were protein-restricted as adults. B) Female flesh flies that are denied a protein meal until days 3, 6, 9, or 12 after eclosion have a similar rate of egg provisioning despite differences in the timing of the availability of protein. Numbers represent means and error bars indicate standard error.

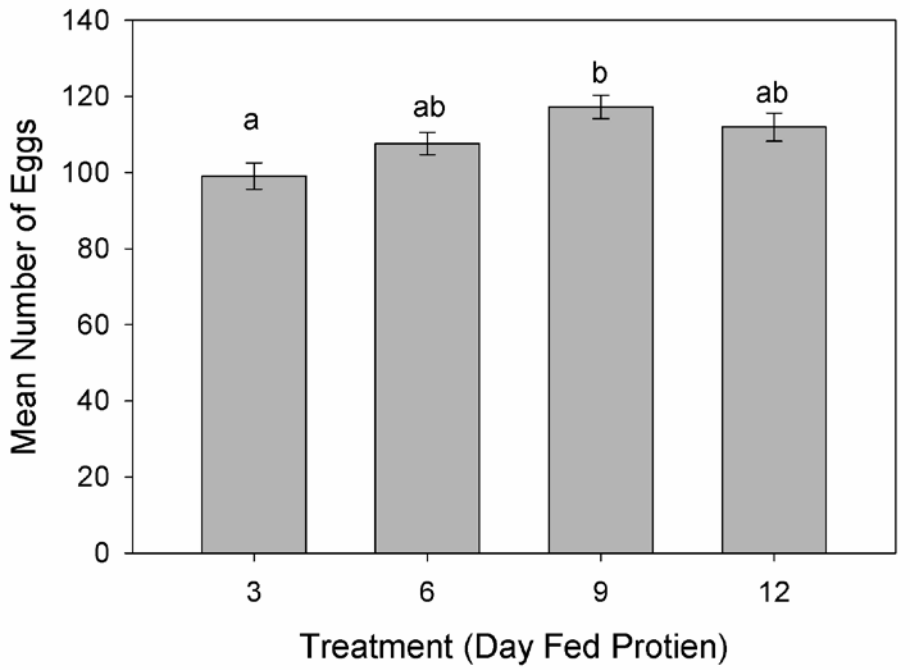

Figure 5-2. Average number of eggs in the first clutch across treatments provided protein on day 3, 6, 9, or 12 after adult eclosion. Bars represent standard error and letters denote statistical separation of means using Tukey's HSD.

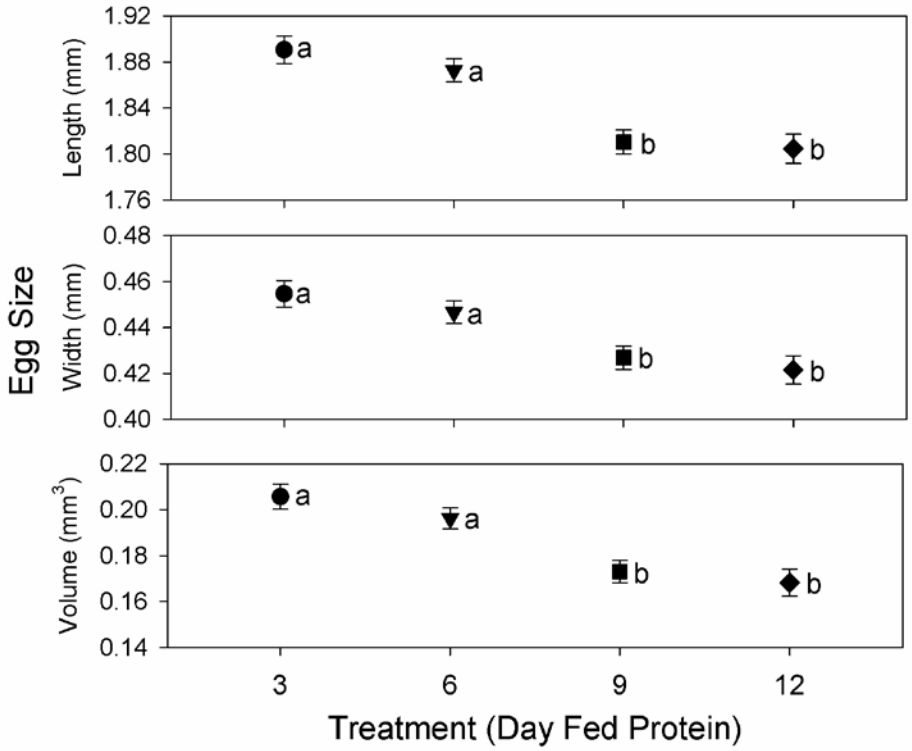

Figure 5-3. Average egg length, width and estimated volume (based on an ellipsoid) showing differences in egg size between flies provided protein on day 3, 6, 9, or 12 after adult eclosion. Flies provided a protein meal within the first week of eclosion have larger eggs than flies that were denied protein up to 12 days post-eclosion. Bars represent standard error and letters denote statistical separation of means using Tukey's HSD.

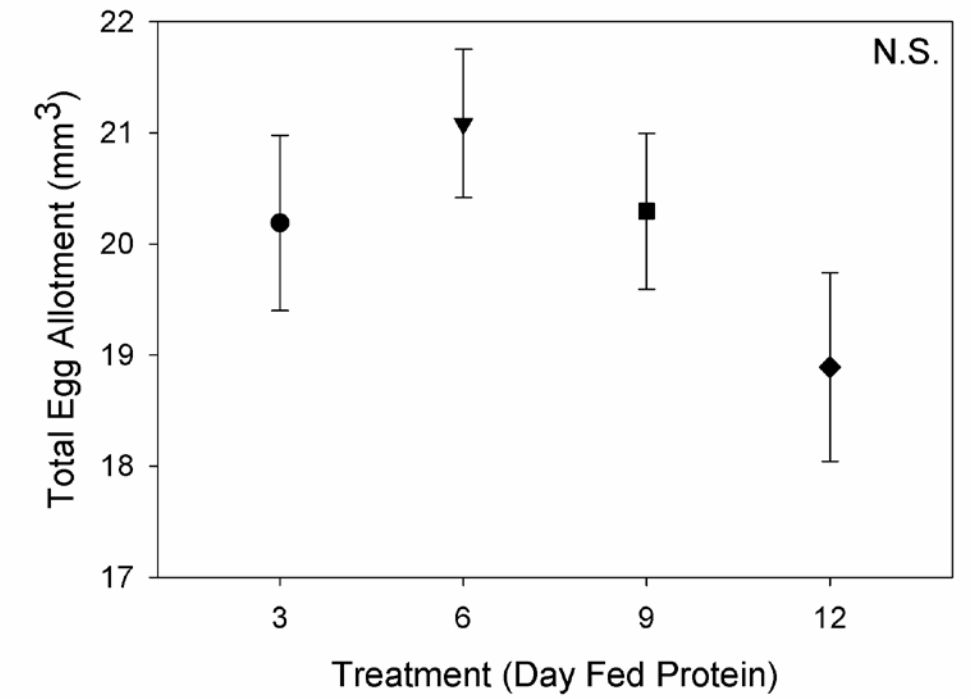

Figure 5-4. Total egg allotment (average egg volume x number of eggs) for flies restricted from protein for 3, 6, 9, or 12 days. N.S. indicates no statistical significance.

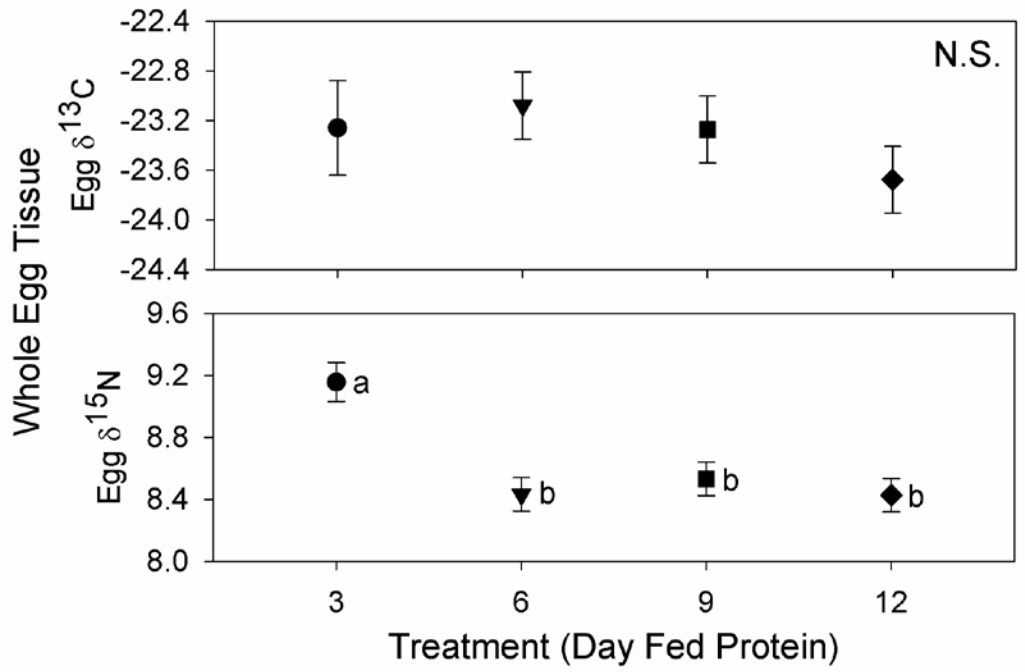

Figure 5-5. Stable isotope profiles for whole egg tissue. There was no statistical significance between egg $\delta^{13}C$ from flies provided protein on day 3, 6, 9, or 13 after adult eclosion. Flies provided protein 3 days post-eclosion had higher $\delta^{15}N$ values than flies that were denied protein until day 6, 9, or 12 after adult eclosion. Error bars represent standard error and letters denote statistical separation of means using Tukey's HSD.

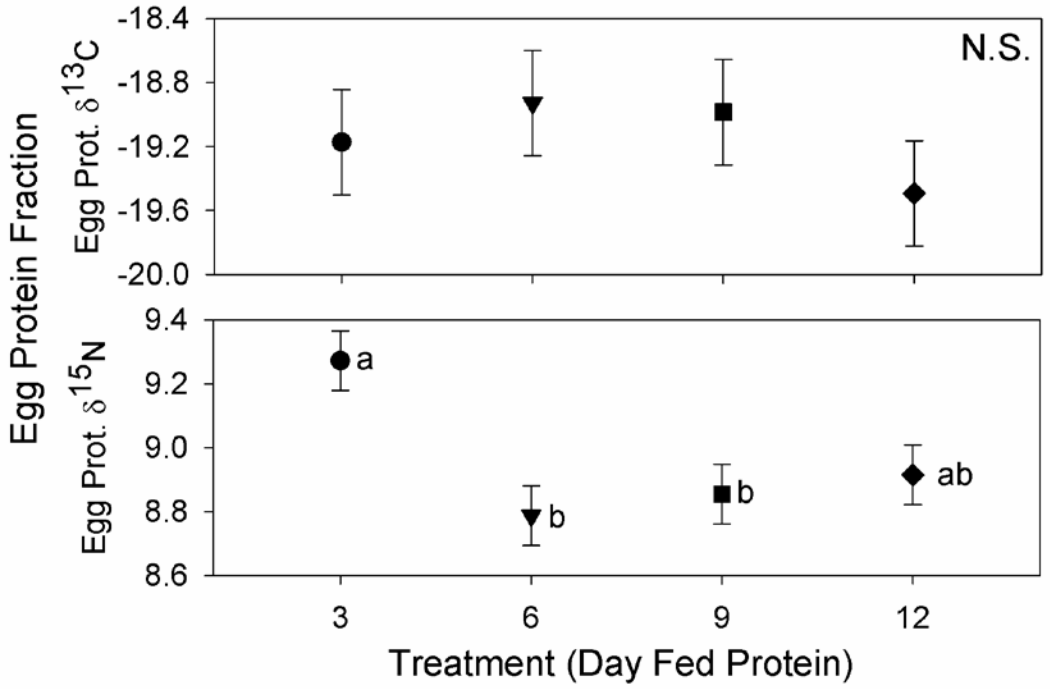

Figure 5-6. Stable isotope profiles for the protein fraction of egg tissue. Similar to whole egg tissue, there was no statistical significance between egg $\delta^{13}C$ between all treatments and flies provided protein 3 days post-eclosion had higher $\delta^{15}N$ values than flies that were denied protein until day 6, 9, or 12 after adult eclosion. Error bars represent standard error and letters denote statistical separation of means using Tukey's HSD

CHAPTER 6
BIOCHEMICAL DISSECTION OF THE METABOLIC RESERVES AND FUEL USE IN THE OVERWINTERING DIAPAUSE OF THE FLESH FLY, *SARCOPHAGA CRASSIPALPIS*.

Introduction

Diapause is an environmentally-programmed life history strategy that allows insects to escape poor environmental conditions, such as drought or winter. There are a wide variety of diapause strategies in insects and examples exist of diapause in nearly every developmental stage, from embryos to adults. However, most species diapause in a specific stage (Denlinger 2002). Diapause can be obligatory, where developmental stasis occurs irrespective of environmental conditions, or it can be facultative, exercised only when necessary to escape adverse environmental conditions. A prerequisite of facultative diapause is the ability to detect and interpret a variety of environmental cues (e.g. day length, temperature, rainfall, etc.) to initiate a genetically programmed "holding pattern" (Harvey 1962, Denlinger 2002). During diapause, many aspects of both development and behavior are suppressed or halted (e.g. growth, reproduction, movement, feeding). Therefore, in addition to monitoring abiotic conditions, diapausing species must have the ability to evaluate internal nutritional conditions to ensure that sufficient resources exist to survive in a suspended state for a long period of time.

During diapause, the efficient utilization of resources is critical; especially in species that diapause in non-feeding stages (i.e. egg and pupal diapausing species). The importance of fuel economy is evident in the characteristic suppression of metabolism and development that coincide with diapause (Hahn and Denlinger 2007). Even with these energy saving tactics, organisms will substantially deplete their metabolic resources during diapause. We often see the consequences of this in the

reduced fecundity and survival of diapausing individuals compared to their non-diapausing counterparts (Denlinger 1981, Ishihara and Shimada 1995, Han and Bauce 1998, Ellers & Van Alphen 2002, Williams et al. 2003, Matsuo 2006). For an organism to successfully survive diapause, they must acquire and store a suitable quantity of resources prior to diapause. This is particularly important in insects that undergo diapause during the pupal stage because they must maintain enough stores to initiate and survive for months without food or water and they must also have enough reserves remaining to be able to re-initiate and complete development then locate food as an adult.

There are several metabolic strategies that pupal-diapausing species employ prior to diapause to increase their chances of survival. First, diapause-destined larvae can increase their nutritional reserves by storing more resources, ultimately obtaining a greater overall mass, as is the case with the cabbage white butterfly, *Pieris rapae*, where diapause-destined larvae are heavier than non-diapausing larvae (Kono 1970). Another strategy is to increase the proportion of metabolic fuels to maximize the storage of energy dense macromolecules (i.e. triacylglycerides), an example of this is the larvae of the pink bollworm, *Pectinophora gossypiella*, where diapause-destined pupae have greater lipid stores than non-diapausing individuals (Adkisson et al. 1963). Finally, individuals can trade-off the increased storage of one class of macromolecules (e.g. lipids) at the cost of another (e.g. carbohydrates). These strategies (greater size, metabolic allometry and trade-offs) are not necessarily the rule, as some diapausing species show no appreciable difference in body composition compared to their non-diapausing counterparts (Saunders 1997, 2000). However, the presence of multiple

strategies for increasing the storage of metabolic fuels indicates that these fuels are an important component of successful diapause.

Insects have three main classes of energy storage molecules, lipids (mostly in the form of triacylglycerides), sugars (which are stored as glycogen or trehalose), and storage proteins (largely hexamerins). All three groups of these storage molecules are commonly associated with diapause (Valder et al. 1969, Adedokun and Denlinger 1986, Siegert 1986, Wipking et al. 1995, Han and Bauce 1998, Zhou and Miesfeld 2009). In addition to their use as metabolic substrates during diapause, some of these molecules can also be used to mechanically protect insects from cold conditions during overwintering diapause (Somme 1982). For example, glycogen can be converted into sorbitol and glycerol, which act as cryoprotectants (Chino 1957, 1958, Somme 1982, Storey 1997). Increased concentrations of free amino acids in the hemolymph can serve as both cryoprotectants and fuel (Boctor 1981, Morgan and Chippendale 1983, Lefevere et al. 1989, Goto et al. 1998). Proteins and sugars clearly play an important role in diapause and further stress the importance of nutritional reserves during diapause and that their utility may not be limited to their use as metabolic substrates.

The ubiquitous nature of diapause in insects suggests that successful diapause can have significant positive effects on fitness. Therefore, understanding how larval nutritional reserves are utilized during diapause is important. Many insect species rely on nutritional reserves acquired as larvae to contribute to life-history traits during adulthood (e.g. reproduction, longevity, etc.). However, diapausing species will deplete a large portion of larval reserves while weathering unfavorable conditions in diapause. Fuel use during diapause is a dynamic process and the metabolic substrate is not

always constant throughout diapause; several species use a combination of metabolic substrates, sometimes switching from one resource pool to another (Adedokun and Denlinger 1985, Han and Bauce 1998, Yocum et al. 2005, Zhou and Miesfeld 2009). A switch in substrates could be a response to one fuel source being depleted or it may indicate a shift in metabolism associated with a change in development or response to the environment (e.g. diapause break, morphogenesis, etc.). However, in pupal-diapausing species the distinction between diapause and post-diapause development is not always clear. Therefore, it is important to accurately determine when diapause ends and post-diapause development begins to separate nutritional changes during diapause from those after diapause termination. Diapause induction and termination have been studied extensively in the flesh fly, *Sarcophaga crassipalpis* Macquart, and both morphological and physiological markers for diapause induction and termination have been described in the literature (Denlinger 1972, Denlinger et al. 1972, Denlinger 1981). Flesh flies also undergo a switch in metabolic substrate, during the first half of diapause lipid mass is rapidly lost; however, it is unknown what substrate is used during the second half of diapause (Adedokun and Denlinger 1985). In this study, we attempted to characterize the metabolic switch identified by Adedokun and Denlinger (1985) during diapause in *S. crassipalpis* by expanding their nutritional analysis to a finer scale by measuring individual metabolic substrate use during diapause. In addition, we went beyond Adedokun and Denlinger (1985) by using respirometric and stable isotope techniques to further characterize the fuel switch in *S. crassipalpis*. We followed a cohort of diapausing flesh flies and took weekly measures of weight, glycogen, protein, glycerol, and neutral lipids (primarily triacylglycerides). In addition, we monitored the

eclosion of a cohort of nearly 300 individuals. We also measured organismal metabolism by taking bi-weekly measurements of respired CO_2 from 100 of these flies. Respiratory metabolism is directly linked to the catabolism of metabolic substrates, which each have distinct stable carbon isotope profiles. To track changes in metabolic substrate catabolism, we took weekly measures of the carbon isotope (^{13}C) profiles in the respired CO_2 of diapausing flies.

Materials and Methods

Insect Rearing and Diapause Initiation

Experiments were conducted using a laboratory colony of *S. crassipalpis* maintained at the University of Florida following the methods of Denlinger (1972). Larvae were reared at a density of 80 individuals per 50 g of beef liver in a 25°C room with a 16: 8 L: D light cycle. Liver was placed in aluminum foil packets that rested on a bed of vermiculite in a plastic container (30 x 15 x 10 cm). After reaching the third instar, larvae wandered out of the foil packets and pupated in the vermiculite. After 5 days at 25°C, the pupae were sifted from the vermiculite and maintained in ventilated cups at 25°C until eclosion. Diapause in *S. crassipalpis* is induced by a combination of photoperiodic cues received in the embryonic stage and temperature cues that the larvae are exposed to (Denlinger 1971). On the day of eclosion, individuals were placed into screened cages (30 x 30 x 30 cm) and incubated at 25°C under a 9: 15 L: D cycle to expose their newly hatched eggs to short day conditions. Once flies were ready to lay their first clutch of larvae, they were provided with 30 g of beef liver in a petri dish as a substrate for laying, which was removed after 6 hours. The resulting first instar larvae were divided among 26 aluminum foil packets at a density of 80 larvae per

50 g of liver. These flies were then placed in an environmental chamber at 20°C with a 9: 15 L: D cycle to program the animals to enter pupal diapause.

Monitoring the Diapause Response

We monitored the eclosion frequency of 288 diapausing *S. crassipalpis* pupae from our diapause cohort. Pupae were placed into the wells of three 96-well plates which were capped with another 96-well plate inverted over the base plate. Both plates were held together with rubber bands and air holes were drilled in the top plate to allow gas exchange. Eclosion was monitored daily for 95 days, after which any remaining pupal cases were opened to determine mortality. The eclosion plates were maintained in a growth chamber at 20°C with a 9:15 L: D cycle.

Micro-Separation and Quantification

Throughout diapause, pupae were sampled every week for a total of 11 weeks. Development was evaluated by gently removing the operculum of the puparium and noting morphological markers associated with diapause. Individuals throughout weeks 1-8 were in diapause, while week 9 individuals were beginning to break diapause (well developed antennal imaginal discs), week 10 individuals were intermediate in their post-diapause pharate adult development (distinct red eyes), and week 11 individuals were sampled late in their post-diapause pharate adult development (with melanized setae visible) (Fraenkel and Hsiao 1968). Metabolic reserves were evaluated by extracting glycogen, protein, and neutral lipids (primarily triglycerides) from 8 individuals each week throughout diapause. Classes of macromolecules were extracted from individual pupae using the micro-separation procedure developed by Van Handel (1965) and modified by Zhou et al. (2004). We were primarily interested in soluble storage proteins and therefore did not extract sugars and individual amino acids in the aqueous phase of

the lipid extraction as in Zhou et al. (2004). The neutral lipids were quantified using the sulpho-phospho-vanillin assay as described in Van Handel (1985). Glycogen concentration was determined with the anthrone assay described in Van Handel (1965). Soluble protein concentration was quantified using a Lowry-type assay, the Bio-Rad DC protein kit (Bio-Rad Laboratories, Hercules, CA, USA).

To determine free glycerol levels, an additional eight pupae were sampled every week for 11 weeks. Samples were lyophilized and homogenized in 2 ml of ultrapure water and 3 µl of the homogenate was used for the glycerol assay. Glycerol levels were determined using an enzyme-based colorimetric assay, the Sigma free-glycerol determination kit (Sigma-Aldrich, St. Louis, MI, USA). Enzyme-grade glycerol (Fisher Scientific, Fair Lawn, NJ, USA) was used as a standard after being diluted in ultrapure water to a concentration of 0.1µg/µl.

Weight Loss and Indirect Calorimetry

A subsample of 100 *S. crassipalpis* pupae was taken from the diapausing cohort to measure metabolic rate (twice weekly) and mass (once weekly) throughout diapause. The diapause duration and initial and final weights through diapause were recorded to test for a relationship between mass, diapause length, and diapause termination. Pupae were maintained in a growth chamber under 20°C short day conditions. Because respirometric gas exchange is representative of whole-organism metabolism, we tracked CO_2 production from 100 pupae throughout diapause. To measure metabolic rate (µl/ hr) we used the manual bolus integration method described by Lighton (2008), where individual pupae were sealed in a 5 ml polypropylene syringe for approximately 6 hours to allow CO_2 levels to accumulate. While sealed, the syringes were held in an air-tight chamber containing soda lime to maintain a CO_2-free

atmosphere around the syringe chambers. After the 6 h seal time, 2 ml of gas from the syringe was injected into a respirometer to quantify respired CO_2, and if the CO_2 volume was large enough, another 2 ml was injected into an air-tight 15 ml conical bottom glass vial for stable isotope analysis. Carbon dioxide concentration was measured with a Li-Cor 7000 differential gas analyzer (Lincoln, NE, USA). Air flow rate was fixed at 150 ml/min using a Sierra instruments mass flow controller (Monterey, CA, USA) paired with a Sable Systems mass flow controller – MFC-2 (Las Vegas, NV, USA). Both gas analyzers were interfaced into a Sable Systems User Interface UI-2 and data was collected and analyzed with Sable Systems Expedata data logging software.

Analysis of ^{13}C in Respired CO_2

For the first ten weeks of diapause, between 6 and 10 respiratory gas samples from the initial cohort of 100 diapausing pupae were analyzed each week to determine the ^{13}C content of the respired CO_2. During initial calibration we determined that the minimum volume of CO_2 per sample needed for accurate isotope analysis was at least 200 µl. Different classes of macromolecules in each organism contain different amounts of ^{13}C and a shift in isotope concentrations of respired CO_2 could indicate a change in metabolic substrate (DeNiro and Epstein 1978). Stable isotope concentration is typically presented in delta notation (i.e. $\delta^{13}C$ for ^{13}C). Delta notation simply represents the ratio of the heavy ^{13}C to the light ^{12}C isotope compared to a standard; they are commonly used in biological studies of resource use and allocation. Several reviews are available to provide more background information on the use of stable isotopes in biological studies (see Gannes et al. 1997, Hood-Nowotny & Knols 2007, Karasov & Martinez del Rio 2007).

The isotopic concentration of respired CO_2 was determined with a Thermo Finnigan DeltaPlus XL isotope ration mass spectrometer (Thermo Fisher Scientific, Duluth, GA, USA). The mass spectrometer was linked to a GasBench II universal on-line gas preparation unit (Thermo Fisher Scientific, Duluth, GA, USA) that was outfitted with a CTC Analytics PAL autosampler (CTC Analytics, Zwingen, Switzerland).

Statistical Analyses

All statistical analyses were performed using the JMP 7 analysis software (SAS Institute, Cary, NC, USA). Analysis of covariance (ANCOVA) was used to assess whether pupal weight or time had an effect on nutritional stores. There was no interaction between weight and time for any of the nutritional stores tested; therefore, data are presented with the interaction term removed from the model. One-way ANOVAs were used to assess differences in pupal weight or nutritional stores (protein, lipid and glycogen) over diapause. Means were separated using Tukey's honestly significant difference with a p value set at 0.05.

Results

Characteristics of Diapause in *S. crassipalpis*

Of the 288 individuals that were monitored for eclosion 76.4 % (n = 220) initiated diapause, while 14.2 % (n = 41) did not diapause and maintained a direct-developmental trajectory and 9.4% (n = 27) did not survive to adult emergence. In this population, the direct-developing flies took 27.4 ± 2.3 (std. dev.) days post-pupariation to develop while the diapausers took 59.4 ± 12.0 days at 20ºC (Figure 6-1A). The metabolic rates (determined by CO_2 production) of ten diapausing and ten non-diapausing flies were followed throughout diapause (Figure 6-1B). Both metabolic trajectories follow the classic U-shaped curve associated with dipteran metamorphosis

and they show the distinct biphasic diapause termination described by Ragland et al. (2009), diapausing flies were more metabolically depressed than their non-diapausing counterparts (Figure 6-1B).

Weight loss throughout diapause was characteristic for flies. There was a large drop in weight after the first week, typical of water loss during pupation, which occurs a few days after pupariation (Figure 6-2). After the first week, weight loss was constant at approximately 1 mg per week until eclosion. There was no relationship between the initial weight of the pupae and the length of diapause. In addition, there was no relationship between the final weight taken prior to eclosion and the length of diapause, indicating that there was no lower weight threshold for diapause termination.

Fuel Use during Diapause

Neutral lipid stores decreased consistently throughout diapause and were almost half depleted by the end of diapause (Figure 6-3A, ANOVA, lipid (mg), $F_{10, 63} = 6.70$, $p < 0.001$). There was no effect of pupal weight on the quantity of neutral lipid stores; however, there was an effect of time in diapause (Table 6-1). In contrast, the protein levels did not change throughout diapause and there was no effect of time in diapause on protein content, although pupal weight did describe some of the variation in protein content (Table 6-1, Figure 3B, ANOVA, protein (mg), $F_{10,66} = 0.39$, $p = 0.949$). Both pupal weight and time in diapause had an effect on glycogen content (Table 6-1). The pattern of glycogen content over time is interesting, because glycogen levels dropped sharply during the weeks following pupariation and began to increase again before dropping immediately before adult eclosion (Table 6-1, Figure 6-3C, ANOVA, glycogen (mg), $F_{10, 77} = 11.96$, $p < 0.001$). In contrast, glycerol concentration did not change much throughout diapause but dropped sharply prior to adult eclosion (Table 6-1, Figure

6-3D, ANOVA, glycerol (mg) $F_{10,77} = 7.73$, $p < 0.001$). While glycerol may be used as a cryoprotectant, we do not see any evidence that glycerol is being converted back into glycogen upon diapause termination, if this were the case, we would expect to see a change in glycerol levels that inversely corresponds to the changes in glycogen levels.

Carbon 13 levels in respired CO_2 did change throughout diapause, suggesting that the metabolic substrates used during diapause are not consistent (ANOVA, $\delta^{13}C$ of exhaled CO_2, $F_{9, 74} = 18.62$, $p < 0.001$). During the first eight weeks of sampling, $\delta^{13}C$ values were relatively low but highly variable (Figure 6-4). On weeks 9 and 10, after the flies had completed diapause and were undergoing metamorphosis, $\delta^{13}C$ values increased and sample variance decreased (Figure 6-4). During post-diapause metamorphosis, cumulative metabolism was much greater than during diapause (Figure 6-1B). The greater concentration of CO_2, likely improved stable isotope detection and contributed to reducing the variability in the data during the last two weeks of sampling.

Discussion

The results suggest that metabolism during diapause is more complex than has been previously reported in the literature. Soluble protein levels did not change throughout diapause, suggesting that soluble protein is not a major metabolic fuel during diapause (Figure 6-3B). However, neutral lipid and glycogen levels fluctuated throughout diapause. Neutral lipids constantly decreased throughout diapause and glycogen levels changed dynamically over the length of diapause (Figures 6-3A and C). Previous work on metabolic reserves associated with diapause in *S. crassipalpis* reported that total lipid decreases rapidly during the first 40 days of diapause but then levels off and does not appreciably decrease during the second half of diapause

(Adedokun and Denlinger 1985). This plateau in lipid loss led Adedokun and Denlinger (1985) to hypothesize that *S. crassipalpis* was primarily utilizing lipids as a metabolic substrate during the first half of diapause and once those lipid stores were depleted, they switched to another metabolic fuel such as proteins or carbohydrates. Our results do not support this fuel-switching hypothesis; we found a constant decrease in neutral lipid concentration during diapause (Figure 6-3A, weeks 1-8) which continued through post-diapause development (Figure 6-3A, weeks 9-11). In addition, glycogen levels, although variable, do not suggest that they are a major metabolic resource for diapause. The discrepancy between lipid levels reported in this study and those reported by Adedokun and Denlinger (1985) may be due to differences in the method of lipid determination between the two studies. First, and most notably, we fractionated neutral lipids from polar lipids in our samples, whereas Adedokun and Denlinger (1985) measured total lipids. Our preliminary data showed that the primary constituent of the neutral lipid fraction in *S. crassipalpis* pupae were triacylglycerides (Wessels, unpublished data), therefore, the neutral lipid fraction is likely more representative of the metabolic substrate than a measurement of total lipids. Next, the sensitivity of the method of lipid determination allowed us to measure neutral lipids levels in individual pupae, whereas the gravimetric-based lipid determination used by Adedokun and Denlinger (1985) pooled samples to obtain 10 g of tissue for accurate analysis. It is likely that these methodological differences contributed to the differences between our findings and those of Adedokun and Denlinger (1985).

Glycogen levels fluctuated throughout diapause and post-diapause development (Figure 6-3C). Glycogen levels initially drop following pupariation and begin to rise

again 7 to 8 weeks after pupariation before declining again in the week prior to eclosion (Figure 6-3C). Several others noted a very similar pattern of glycogen depletion and accumulation during insect diapause (Chino 1958, Adedokun and Denlinger 1985, Rickards et al. 1987). In insects, glycogen is the primary precursor to the cryoprotectant polyols glycerol and sorbitol (Chino 1958, Wyatt 1967, Tsumuki et al. 1987, Storey 1997). Lee et al. (1987) reported that glycerol is the primary low molecular weight polyol found in high concentrations in diapausing *S. crassipalpis* pupae. Because of the relationship between glycogen and polyol levels, many insects have an inverse relationship in the quantity of these molecules present during diapause (Chino 1958, Tsumuki et al 1987). However, we found that there was no significant difference in glycerol levels throughout diapause (Figure 6-3D, weeks 1-8), although, glycerol levels did decrease in the weeks before adult emergence during post-diapause development (Figure 6-3D, weeks 9-11). Our findings contrast with those of Lee et al. (1987), who found that under similar rearing conditions (diapausing flesh flies maintained at 20ºC) glycerol levels in *S. crassipalpis* increased significantly throughout diapause before dropping rapidly during post-diapause development. The findings of Lee et al. (1987) are more in accord with what we would expect if glycogen was being converted to glycerol during diapause and back into glycogen upon the termination of diapause.

The ^{13}C profiles of exhaled CO_2 confirm our findings that metabolism during diapause is a dynamic process. Respiratory stable isotopes have been used to detect shifts in the metabolic fuel of hummingbirds (Carleton et al. 2006, Welch and Suarez 2007). In addition, the value of CO_2 stable isotope measurements in characterizing insect diet has been known for quite some time (DeNiro and Epstein 1978). The stable

isotope data support our biochemical data that indicate there is not a distinct shift in metabolic substrate use during diapause (Figure 4). However, the respired isotope profiles suggest that there are differences between fuel metabolism during diapause (Figure 6-4, weeks 1-7) and during post-diapause development (Figure 6-4, weeks 8-10). During diapause, the stable isotope data were quite variable, however most weeks had significantly lower $\delta^{13}C$ values than the values recorded during post-diapause development on weeks 9 and 10 (Figure 6-4). In this study, neutral lipid extracts were relatively deplete in ^{13}C, $\delta^{13}C = -20.14 \pm 0.22$ ($n = 12$) compared to published values of *S. crassipalpis* tissue (Wessels et al. 2010a), and the $\delta^{13}C$ values of respired CO_2 hovered around this level during diapause, indicating that neutral lipids are being metabolized during diapause. However, following diapause break (Figure 6-4, week 7), respired $\delta^{13}C$ values begin to rise and peak during the last 2 weeks of post-diapause development prior to adult emergence. This suggests that in addition to lipids, other fuels are being metabolized during post-diapause development. This hypothesis is also supported by the biochemical data, during the last three weeks of diapause, neutral lipid levels decline significantly (Figure 6-3A) and glycogen and glycerol levels also drop significantly in the week preceding adult emergence (Figure 6-3C and 6-3D). Isotope values for lipid, glycogen and soluble protein fractions have been determined in both bottle flies and house flies (DeNiro and Epstein 1978). When these flies were reared on diets of horsemeat (low $\delta^{13}C$) and pork (high $\delta^{13}C$), their glycogen fractions contained more ^{13}C than the lipid fractions despite differences in dietary ^{13}C content (DeNiro and Epstein 1978). However, DeNiro and Epstein (1978) caution that the differences between biochemical fractions in animals and their diets can vary based on many

factors (e.g. $\delta^{13}C$ of the diet, biochemical composition of the diet, amount of *de novo* biochemical synthesis in the animal, etc.). A similar relationship between glycogen and lipid was found in the ^{13}C signatures of songbird tissue fractions raised on high and low $\delta^{13}C$ diets (Podlesak et al. 2005). In both examples, the $\delta^{13}C$ values of carbohydrates are greater than lipids, suggesting that regardless of dietary $\delta^{13}C$ content, carbohydrates contain more ^{13}C than lipids. If lipid is the primary fuel during diapause, the shift in $\delta^{13}C$ values of respired CO_2 after diapause might represent the increased use of carbohydrates during post-diapause development (Figure 6-4).

While our results did not support the hypothesis that *S. crassipalpis* is consecutively switching fuels during diapause, the hypothesis has merit. Other insect species have been known to undergo sequential shifts in metabolic substrate during diapause. For example, the spruce budworm, *Choristoneura fumiferana*, experiences plasticity in the initiation of larval diapause, individuals that initiate diapause early will likely experience more days at high temperature, which can lead to a heavy loss of metabolic reserves (Han and Bauce 1998). Han and Bauce (1998) showed that the more warm days spruce budworm were exposed to early in diapause, the more lipid reserves were depleted during diapause. In addition, they found that glycogen levels did not significantly drop unless lipid reserves were highly depleted. Based on these findings, Han and Bauce (1998) hypothesized that lipids were a major fuel source for diapause and when lipid stores were depleted, spruce budworm would shift to using glycogen as a metabolic fuel. Similar to the spruce budworm, the solitary bee, *Megachile rodtunda*, also seems to switch fuels from lipid to carbohydrate during diapause. Instead of directly quantifying metabolic reserves, Yocum et al. (2005)

monitored respiratory metabolism in *M. rotunda*. During the first three months of diapause, the bee had a respiratory quotient (RQ) close to 0.7, which is indicative of lipid metabolism (Yocum et al. 2005). However, in the fourth month of diapause, RQ values shift towards 1.0 for the next three months, which suggests that primarily carbohydrates are being metabolized. In the final month of diapause, *M. rotunda* has a RQ of 0.8, suggesting that a mixture of metabolic resources is being catabolized (Yocum et al. 2005). An opposite fuel switch occurs in the mosquito *Culex pipiens*, which undergoes diapause in the adult stage. Zhou and Miesfeld (2009) provided mosquitoes with a pulse of ^{14}C-labled glucose. They found that that glycogen levels began to decrease early in diapause (14 days) and glycogen levels stopped decreasing and plateau after approximately 35 days of diapause (Zhou and Miesfeld 2009). In contrast, lipid levels remained steady early in diapause and did not begin to decrease until approximately 35 days in diapause (Zhou and Miesfeld 2009). These results suggest that *Cx. pipiens* begins diapause burning primarily glycogen, and when these stores are depleted, they shift to lipid metabolism for the duration of diapause.

Clearly, fuel use during diapause is dynamic and a variety of strategies have been employed by insects to maximize survival and post-diapause success. Understanding the various ways insects cope with diapause is important and has major implications for the evolution of insect life-histories. Studying insect overwintering at the physiological level may also have important ecological and economic implications, ranging from determining and predicting the consequences of climate change to potential for developing novel control methods for agriculturally and medically important

pest species. Future work will continue to focus on the physiological mechanisms underlying diapause and their effects on diapause energetic.

Acknowledgements

We thank Kathy Milne for her assistance rearing and maintaining the laboratory colonies of *S. crassipalpis*. We also thank the HHMI Group Advantaged Training of Research Program for providing financial support to incorporate an undergraduate scholar, Diana C. Jordan, in the design and execution of this project. This work was supported by NSF-IOS-641505 and the Florida State Agricultural Experiment Station.

Table 6-1. ANCOVA tables for the effects of pupal weight and time in diapause on lipid, protein and glycogen stores, stars indicate statistical significance.

Resource Pool	Source	df	F	p
Neutral Lipid	Whole Model	11	6.19	<0.001
	Weight (mg)	1	1.20	0.317
	Time (weeks)	10	4.73	<0.001
	Error	62		
	Total	73		
Protein	Whole Model	11	0.88	0.565
	Weight (mg)	1	5.55	0.022
	Time (weeks)	10	0.38	0.952
	Error	65		
	Total	76		
Glycogen	Whole Model	11	12.59	<0.001
	Weight (mg)	1	8.02	0.006
	Time (weeks)	10	12.92	<0.001
	Error	76		
	Total	87		
Glycerol	Whole Model	11	7.52	<0.001
	Weight (mg)	1	3.21	0.077
	Time (weeks)	10	6.02	<0.001
	Error	76		
	Total	87		

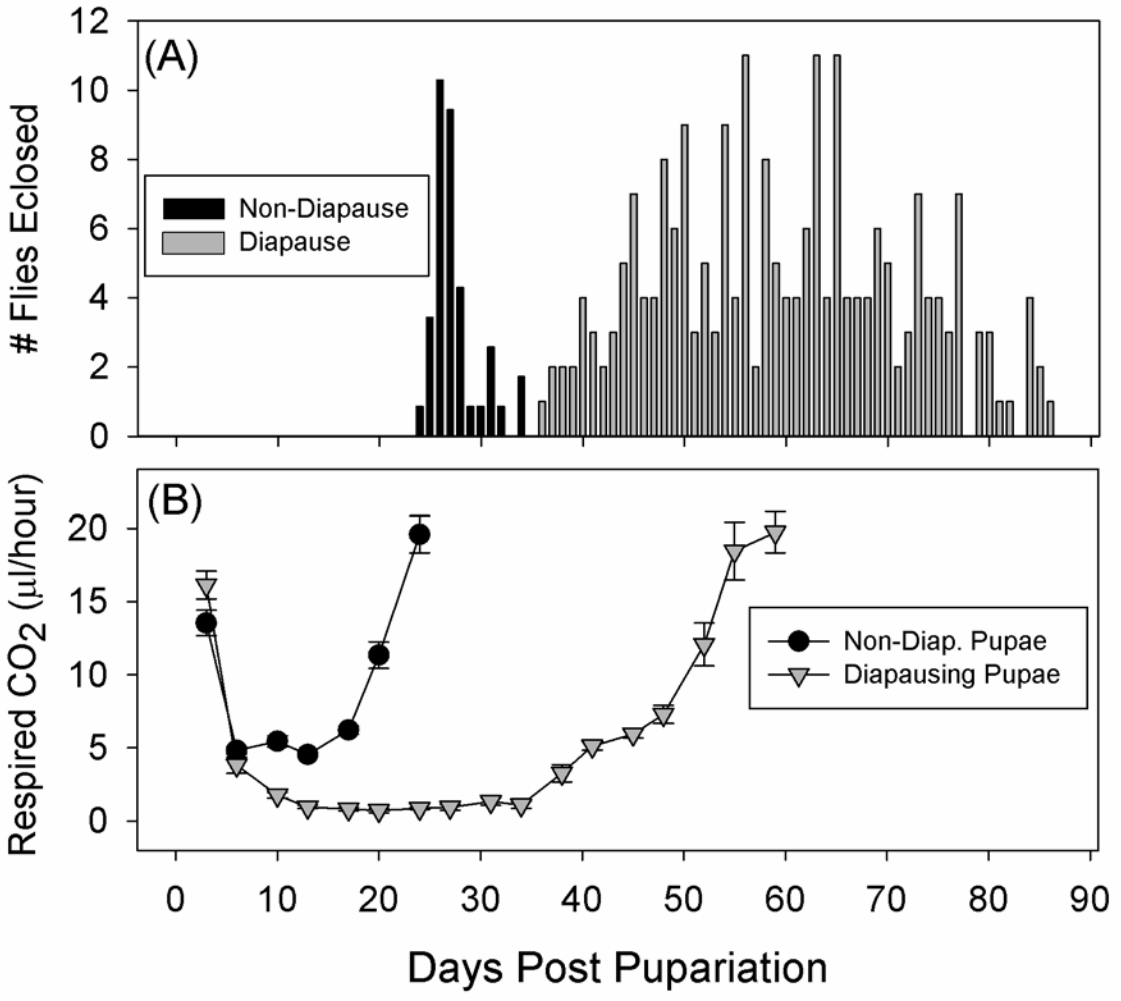

Figure 6-1. A) Eclosion histogram for a cohort of 288 *S. crassipalpis* flesh flies reared under diapause-inducing short-day conditions 14.2 % of the cohort developed normally and did not diapause (black bars) while 76.4 % entered diapause (gray bars) and 9.4 % did not survive pupation (not shown). B) Pupal metabolism depicted as respired CO_2 (µl/hr) for non-diapausing (black circles, n = 10) and diapausing pupae (grey triangles n=10). Values are represented as means ± standard error.

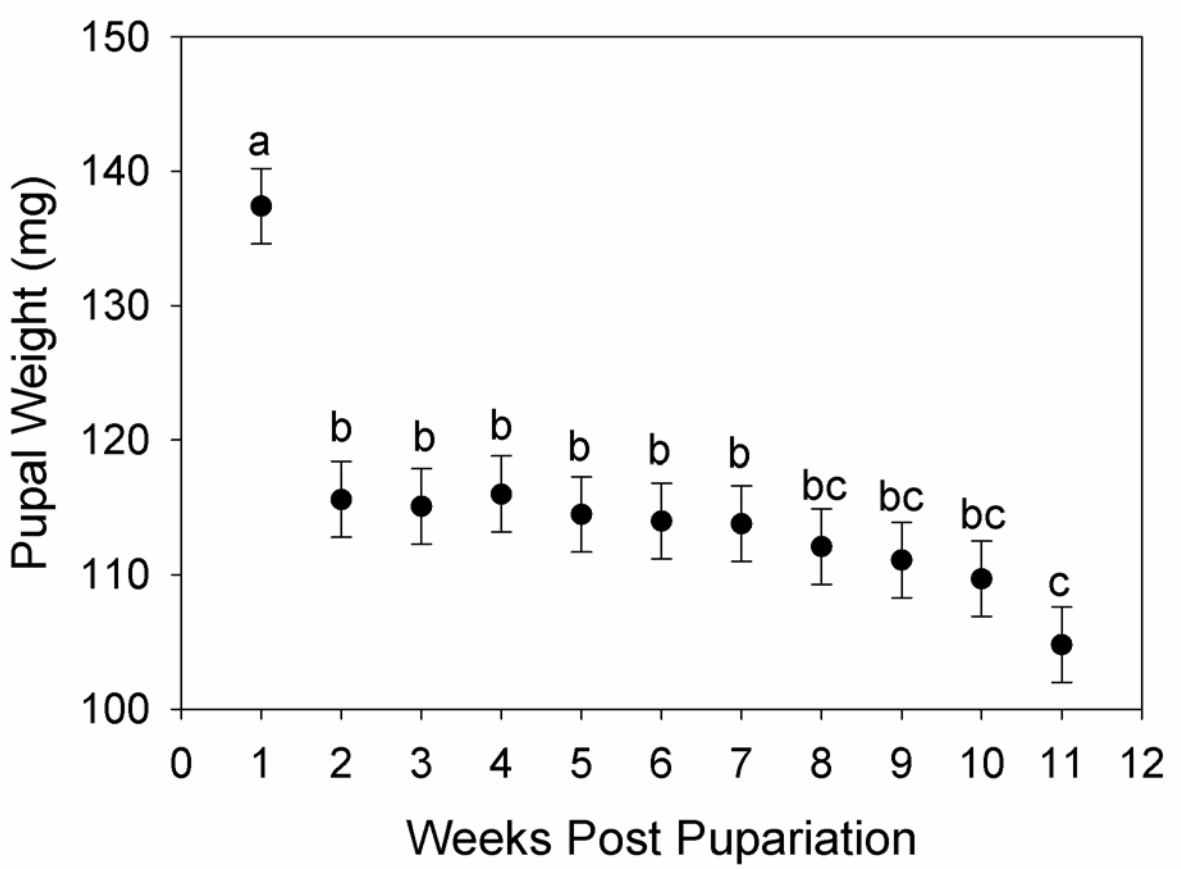

Figure 6-2. Wet mass (mg) of diapausing *S. crassipalpis* pupae across 11 weeks of pupal development. During weeks 1-8 the flies were in diapause, whereas weeks 9-11 are post-diapause development. Values are represented as means ± standard error and letters denote separation of means using Tukey's honestly significant difference.

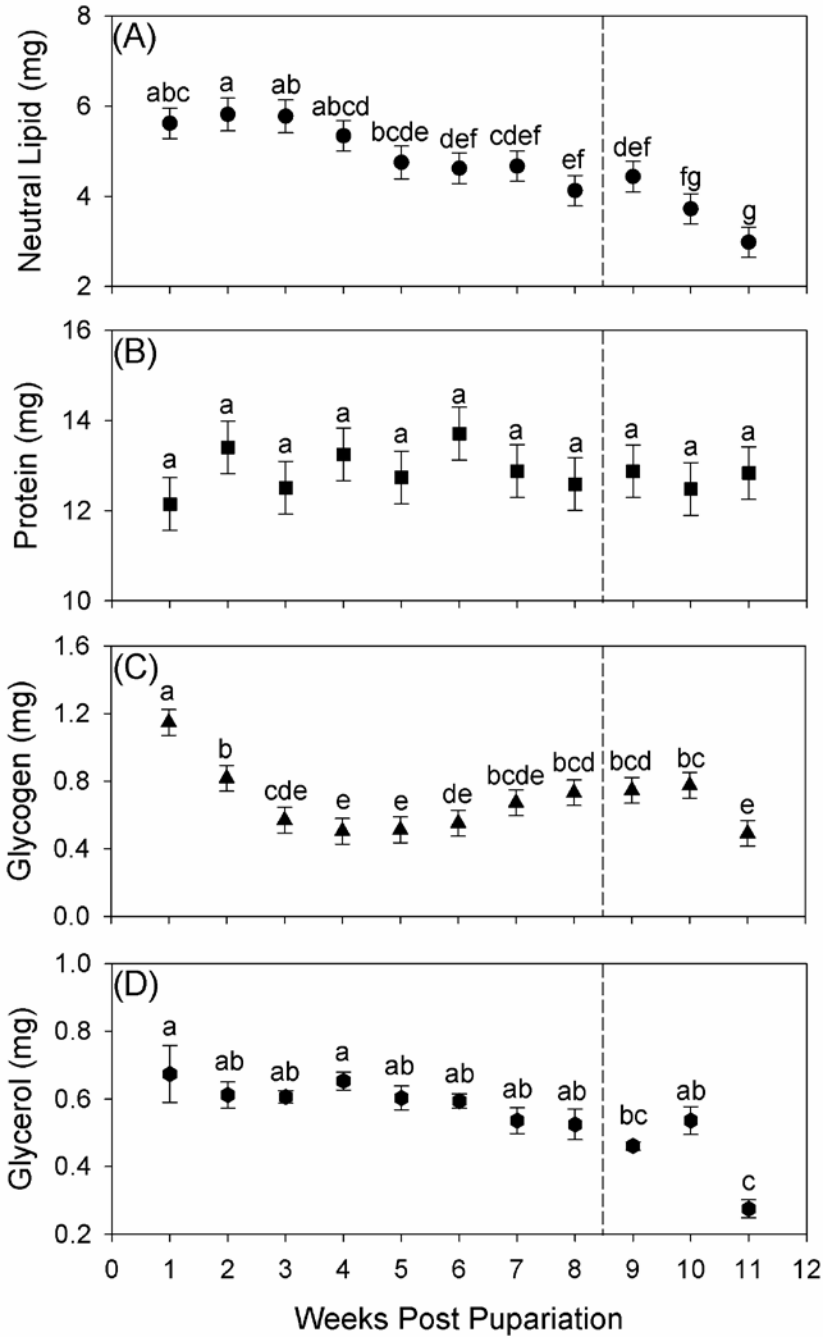

Figure 6-3. Metabolic reserves during diapause and post diapause development (diapause termination is indicated by the vertical dashed line) A) neutral lipids (primarily triacylglycerides) (mg) B) protein (mg) C) glycogen (mg) and D) glycerol (mg) of diapausing *S. crassipalpis* pupae over 11 weeks of pupal development. During weeks 1-8 the flies were in diapause, whereas weeks 9-11 are post-diapause development. Values are represented as means ± standard error and letters denote separation of means using Tukey's honestly significant difference.

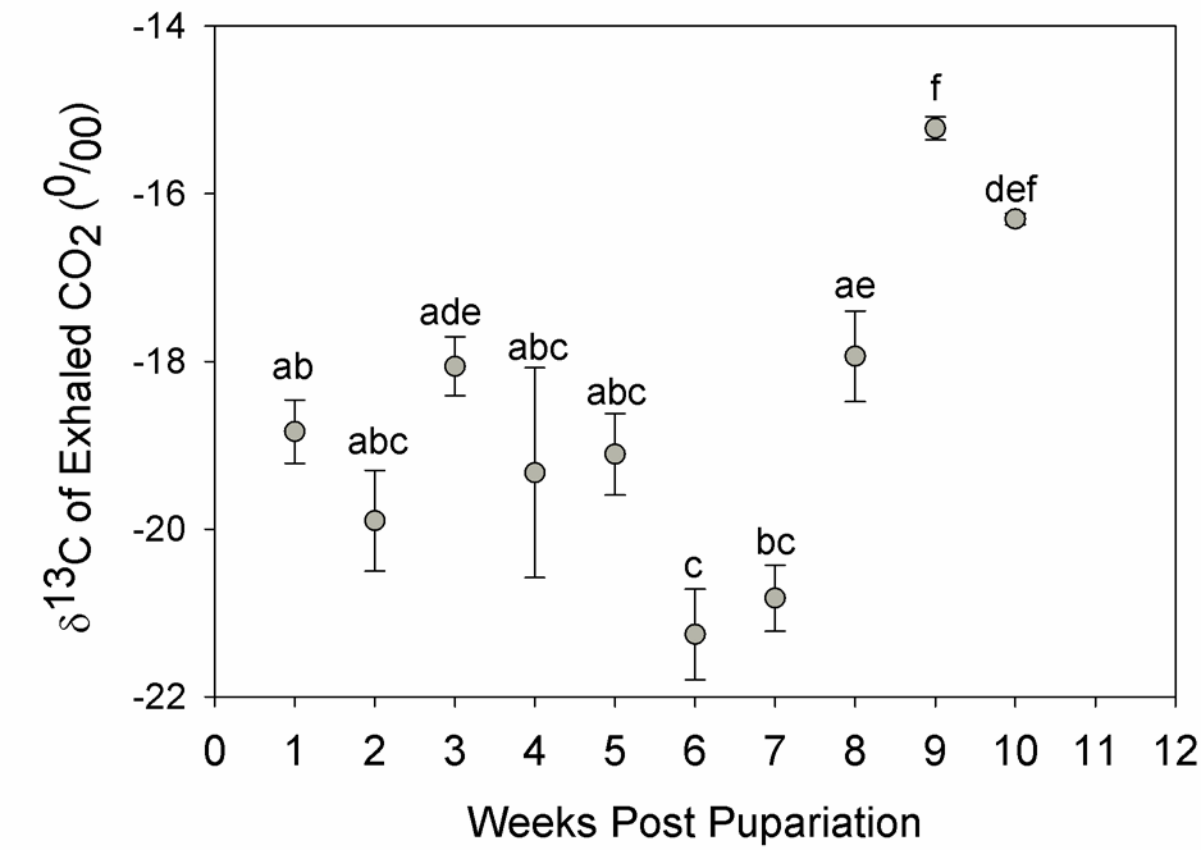

Figure 6-4. Concentration of ^{13}C isotopes in the respired CO_2 of diapausing *S. crassipalpis* over 10 weeks of pupal development. During weeks 1-7 the flies were in diapause, whereas weeks 8-10 are post-diapause development. Isotope levels are represented as means ± standard error in delta notation (see methods for details) and letters denote separation of means using Tukey's honestly significant difference.

CHAPTER 7
OVERALL DISCUSSION AND CONCLUSIONS

My central hypothesis was that resource use and allocation would be flexible and respond to maximize fitness in variable environments. As I will elaborate later, this hypothesis was not necessarily supported by the data. Flesh flies actually had little capacity for plasticity in reproductive allocation from capital and income stores. In hindsight, this is likely due to the unpredictability of resource acquisition associated with the scavenging lifestyle of *S. crassipalpis*. Because these flies both feed and oviposit on carrion, adult resource acquisition is intimately linked to oviposition site availability. My original hypothesis predicts that when flies are faced with lower quality resources, they will sacrifice somatic tissues to compensate for this shortcoming. However, in the field flies exposed to low quality resources will likely also face a low quality or availability of oviposition sites. In this scenario maximizing reproduction would be illogical because it is highly likely that those offspring will not survive to maturity.

The common methodological link between all of the studies presented in this dissertation is the use of stable isotopes to elucidate resource metabolism and allocation. The real power of this approach is the ability to confidently follow the allocation of resources to specific resource pools. While several studies of resource allocation and life-history trade-offs have been conducted before, the majority of those studies have relied on indirect metrics of allocation, measuring increases in tissue mass or egg mass to infer allocation. Some experimental methods such as radioisotopes also enable tracking allocation, they are most commonly used in a “pulse-chase” fashion. Also, when they are administered orally, they are often not uniformly incorporated into the diet. While effective and appropriate for many experimental designs, because of

these limitations, radioactive tracers are not a quantitative tool for studying resource allocation. In addition, one assumption of the pulse-chase experimental design is that resource allocation to a specific pool is constant, however, because nutrient acquisition and metabolism are dynamic processes this assumption may not always be true.

The dynamic nature of metabolism is one of the largest problems with stable isotopes because the heavier molecular weight of ^{13}C and ^{15}N cause subtle changes in the molecular structure of organic molecules (differences in bond strength). When molecules are being constructed, transported and disassembled inside complex multicellular organisms, the molecular subtleties of stable isotopes lead to larger scale differences in abundance (fractionation or bioaccumulation) known as isotopic discrimination. While the fractionation problem is not unique to stable isotopes (the same principles also influence heavier radioactive isotopes), in biological systems fractionation must be accounted for in calculations of isotopic incorporation. Several authors have realized this issue and have indicated a need for more controlled laboratory studies on the dynamics of stable isotope metabolism in biological systems (Gannes et al. 1997, Martinez del Rio et al. 2009). Chapter 2 answers the call for more laboratory experiments put forth by Gannes et al. (1997) by testing the hypothesis that there is a concentration-dependent relationship between the dietary concentration of ^{13}C and ^{13}C fractionation (proposed by Caut et al. 2008). To reduce the complexity in the experimental design I looked for the simplest biological system possible to characterize levels of ^{13}C in different classes of cellular components. I selected the bacterium *B. subtilis* as an experimental organism because it was unicellular and possessed sucrase enzymes, enabling them to thrive in a simple minimal bacterial broth composed of

sucrose, lysine and a combination of salts, where the vast majority of carbon in the broth was derived from sucrose. By mixing cane (C_4) and beet (C_3) sugars, a series of broths were created that had a linear range of ^{13}C concentrations ranging from high to low (chapter 2, Table 2-1). Subsequent analysis of whole bacterial tissue and lipid extracted tissue showed a slight concentration-dependent relationship to dietary ^{13}C level (Figure 2-1, Table 2-2). However, the discrimination between the diet and the lipid fraction was heavily dependent upon dietary ^{13}C concentration. These findings not only support the concentration-dependent discrimination hypothesis of Caut et al. (2008), but they also provide a mechanism, the *de novo* synthesis of lipids, as the minimal broths contained no fatty acids to contribute to the lipid fraction via metabolic routing. These findings contribute to our understanding of the dynamics of stable isotope metabolism and will likely improve the modeling of isotopic fractionation in biological systems.

Many of the results from the studies presented in this dissertation may seem intuitive, for example, the finding that reproductive resources in flesh flies are primarily from adult-acquired resources. Without stable isotopes, I could not determine definitively which resource pools were responsible for egg provisioning. Biologically, it was known that oocyte development in *S. crassipalpis* did not begin until after adult eclosion, and that the quality of adult diet influenced reproduction (Hahn et al. 2008a, Hahn et al. 2008b). However, by using stable isotopes as metabolic tracers, I was able to precisely characterize the roles of capital and income to reproductive allocation in *S. crassipalpis* (chapter 3) and to learn more about allocation under variable nutritional environments. In chapter 4, I tested the hypothesis proposed by Hahn et al. (2008a) that the plasticity in reproductive timing based on the post-threshold availability of

nutrients is an adaptive response to lengthen foraging time. The results of the study supported the adaptive plasticity hypothesis showing that flies provided resources acquired during the reproductive delay reproduced faster and had more eggs and larger eggs than those that did not receive additional protein (Figure 4-2 and 4-3). In addition, beef liver with a unique stable isotope profile was used to track the incorporation of the pulsed resources into eggs. This technique showed that the pulsed resources provided during the reproductive delay were able to be used by the female and linked the phenotypic response (increased fecundity) to the environmental variable (pulsed resources) (Figure 4-4). While this evidence supported the adaptive delay hypothesis in Hahn et al. (2008a), the case for adaptive plasticity is not conclusive. An alternative hypothesis for the reproductive delay that could not be tested by the experimental design of chapter 4, is that the reproductive delay is caused by the reallocation of somatic resources from other pools (i.e. flight muscle, fat body, etc.) leads to a physiological delay in oocyte development. This alternative hypothesis was tested in chapter 5, where adult females were restricted from protein for 3, 6, 9, or 12 days to determine whether longer protein restriction would increase the allocation of somatic resources to reproduction. Adult protein restriction had an effect on reproductive allotment and timing (Figures 5-2, 5-3, and 5-4), but not on the rate of oocyte development (Figure 5-1). I used isotopically distinct diets to distinguish adult and larval acquired resources and found no difference in the proportion of larval-acquired (somatic) resources to eggs, suggesting that *S. crassipalpis* has little capacity for plasticity in reproductive allocation from capital vs. income (Figure 5-5).

The final research chapter of this dissertation, chapter 6, departed slightly from the reproductive theme of the previous three chapters. Chapter 6 focused on the pupal diapause of *S. crassipalpis*, specifically the metabolic substrates used to fuel diapause in this species. Diapause in *S. crassipalpis* has been investigated thoroughly in the literature, perhaps this is due to the interesting peculiarities of diapause in this species. Diapause in *S. crassipalpis* can be artificially terminated by the topical application of organic solvents such as hexane and by the injection of the signaling molecule cyclic GMP (Denlinger and Wingard 1978, Denlinger et al. 1980). Another interesting aspect of *S. crassipalpis* diapause is the presence of infradian cycles of oxygen consumption that are curiously similar to the periodical arousals of hibernating ground squirrels, raising questions about their metabolic significance (Denlinger et al. 1972, Denlinger & Tanaka 1989, Daan et al. 1991). These examples along with other contributions in the insect diapause literature suggest that metabolism during diapause might be more dynamic than previously thought (Hahn and Denlinger 2007). The focus of chapter 6 was to build upon the foundation of diapause metabolism in *S. crassipalpis* initially constructed by Adedokun and Denlinger (1985). Adedokun and Denlinger (1985) found that flesh flies in diapause lost lipid content quickly during the first half of diapause and during the last half of diapause, the decrease in lipid mass began to plateau. Because of these data, it was hypothesized that *S. crassipalpis* primarily used lipid during the first half of diapause and then switched to an unidentified substrate after lipid stores were depleted (Adedokun and Denlinger 1985). To test this hypothesis, I analyzed levels of storage lipids, protein, glycogen, and glycerol over the length of diapause and post-diapause development (chapter 6). However, the methods used to extract, separate

and analyze the metabolic substrates were more sensitive and allowed the analysis at the individual insect level than the methods used by Adedokun and Denlinger (1985). Surprisingly, the results of the metabolic substrate analysis did not support the substrate-shift hypothesis (Figures 6-3 and 6-4). In contrast, the biochemical data along with analysis of stable isotopes in respired CO_2, suggested that lipid metabolism is a large component of diapause metabolism, although other metabolites may be used during this time as well. I also found that during post-diapause development, substrate metabolism shifts to the metabolism of a variety of fuels prior to adult eclosion (chapter 6, Figures 6-3 and 6-4). While contradicting the published literature, these findings suggest that there is more to the story of diapause metabolism in *S. crassipalpis* than was previously thought. Future studies should focus on the intricacies of diapause metabolism, specifically metabolism during the infradian cycles of oxygen consumption to elucidate the functional role of these metabolic oscillations.

In conclusion, this dissertation makes several valuable contributions to a range of fields of inquiry, ranging from stable isotope metabolism to reproductive plasticity and diapause physiology. These studies use stable isotopes to directly link resource acquisition and allocation, a link that was one of the fundamental pieces missing from many studies of life-history evolution. Because life-histories are ultimately a product of intermediary metabolism, the ability to understand how nutritional resources flux through organisms opens a new chapter into the mechanisms that constrain or facilitate life-history evolution. These studies shed light onto the nutritional consequences of environmental variation, and will ultimately make pinpointing the mechanisms

underlying these allocation decisions more feasible by identifying phenotypic changes that were previously undetectable.

LIST OF REFERENCES

Abney, M. R., C. E. Sorenson, F. Gould, and J. R. Bradley. 2008. Limitations of stable carbon isotope analysis for determining natal host origins of tobacco budworm, *Heliothis virescens*. Entomologia Experimentalis et Applicata 126:46-52.

Abraham, W. R., C. Hesse, and O. Pelz. 1998. Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. Applied and Environmental Microbiology 64:4202-4209.

Adedokun, T. A., and D. L. Denlinger. 1985. Metabolic reserves associated with pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. Journal of Insect Physiology 31:229-233.

Adkisson, P. L., R. A. Bell, and S. G. Wellso. 1963. Environmental factors controlling the induction of diapause in the pink bollworm, *Pectinophara gossypiella* (Saunders) Journal of Insect Physiology 9:299-310.

Agrell, J., J. O. Wolff, and H. Ylonen. 1998. Counter-strategies to infanticide in mammals: costs and consequences. OIKOS 83:507-517.

Atlas, R. M., and L. C. Parks. 1997, Handbook of Microbiological Media. Boca Raton, CRC Press.

Barnes, C., C. J. Sweeting, S. Jennings, J. T. Barry, and N. V. C. Polunin. 2007. Effect of temperature and ration size on carbon and nitrogen stable isotope trophic fractionation. Functional Ecology 21:356-362.

Barrett, E. L. B., R. F. Preziosi, A. J. Moore, and P. J. Moore. 2008. Effects of mating delay and nutritional signals on resource recycling in a cyclically breeding cockroach. Journal of Insect Physiology 54:25-31.

Bauerfeind, S. S., and K. Fischer. 2005. Effects of food stress and density in different life stages on reproduction in a butterfly. OIKOS 111:514-524.

Bederman, H. R., D. A. Dufner, J. C. Alexander, and S. F. Previs. 2006. Novel application of the "doubly labeled" water method: measuring CO_2 production and the tissue-specific dynamics of lipid and protein in vivo. AJP - Endocrinology and Metabolism 290:E1048-E1056.

Ben-David, M., T. A. Hanley, D. R. Klein, and D. M. Schell. 1997. Seasonal changes in diets of coastal and riverine mink; the role of spawning Pacific salmon. Canadian Journal of Zoology 75:803-811.

Berrigan, D. 1991. Lift production in the flesh fly, *Neobellieria* (= *Sarcophaga*) *bullata* Parker. Functional Ecology 5:448-456.

Bertness, M. D. 1981. Pattern and plasticity in tropical hermit crab growth and reproduction. The American Naturalist 117:754-773.

Bluthgen, N., G. Gebauer, and K. FIedler. 2003. Disentangling a rainforest food web using stable isotopes: dietary diversity in a species-rich ant community. Oecologia 137:426-435.

Boctor, I. Z. 1981. Changes in the free amino acids of the hemolymph of diapause and non-diapause pupae of the cotton bollworm, *Heliothis armigera* Hbn. (Lepidoptera: Noctuidae). Experientia 37:125-126.

Boggs, C. L. 1997. Dynamics of reproductive allocation from juvenile and adult feeding: radiotracer studies. Ecology 78:192-202.

Boggs, C. L 1997a. Reproductive allocation from reserves and income in butterfly species with differing adult diets. Ecology 78:181-191.

Boggs, C. L 2009. Understanding insect life histories and senescence through a resource allocation lens. Functional Ecology 23:27-37.

Boggs, C. L., and C. L. Ross. 1993. The effect of adult food limitation on life history traits in *Speyeria mormonia* (Lepidoptera: Nymphalidae). Ecology 74:433-441.

Bonnet, X., D. Bradshaw, and R. Shine. 1998. Capital vs. income breeding: and ectothermic perspective. OIKOS 83:333-342.

Bontemps, A., D. Bourguet, L. Pelozuelo, M. T. Bethenod, and S. Ponsard. 2004. Managing the evolution of *Bacillus thuringiensis* resistance in natural populations of the European corn borer, *Ostrinia nubilalis*: host plant, host race and pherotype of adult males at aggregation sites. Proceedings: Biological Sciences 271:2179-2185.

Bradshaw, W. E., and K. Johnson. 1995. Initiation of metamorphosis in the pitcher-plant mosquito: effects of larval growth history. Ecology 76:2055-2065.

Brockelman, W. Y. 1975. Competition, the fitness of offspring, and optimal clutch size. The American Naturalist 109:677-699.

Brown, G. P., and R. Shine. 2002. Reproductive ecology of a tropical natricine snake, *Tropidonophis mairii* (Colubridae). Journal of the Zoological Society of London 258:63-72.

Bylemans, D., D. Borovsky, D. F. Hunt, J. Shabanowitz, L. Grauwels, and A. De Loof. 1994. Sequencing and characterization of try sin modulating ecstatic factor (TMOF) from the ovaries of the grey fleshly, *Neobellieria* (*Sarcophaga*) *bullata*. Regulatory Peptides 50:143-149.

Carleton, S. A., B. H. Bakken, and C. Martinez del Rio. 2006. Metabolic substrate use and the turnover of endogenous energy reserves in broad-tailed hummingbirds (*Selasphorus platycercus*). The Journal of Experimental Biology 209:2622-2627.

Caut, S., E. Angelo, and F. Courchamp. 2009. Variation in discrimination factors (Δ^{15}N and Δ^{13}C): the effect of diet isotopic values and applications for diet reconstruction. Journal of Applied Ecology 46:443-453.

Caut, S., E. Angulo, and F. Courchamp. 2008. Caution on isotopic model use for analyses of consumer diet. Canadian Journal of Zoology 86:438-445.

Cerling, T. E., and J. M. Harris. 1999. Carbon isotope fractionation between diet and bioapatite in ungulate mammals and implications for ecological and paleoecological studies. Oecologia 120:347-363.

Chino, H. 1957. Conversion of glycogen to sorbitol and glycerol in the diapause egg of the *Bombyx* silkworm. Nature 180:606-607.

Chino, H. 1958. Carbohydrate metabolism in the diapause egg of the silkworm, *Bombyx mori*-II. Journal of Insect Physiology 2:1-12.

Chown, S. L., and S. W. Nicholson. 2004, Insect Physiological Ecology. Oxford, Oxford University Press.

Dalerum, F., and A. Angerbjorn. 2005. Resolving temporal variation in vertebrate diets using naturally occurring stable isotopes. Oecologia 144:647-658.

DeNiro, M. J., and S. Epstein. 1977. Mechanism of carbon isotope fractionation associated with lipid synthesis. Science 197:261-263.

DeNiro, M. J., and S. Epstein. 1978. Influence of diet on the distribution of carbon isotopes in animals. Geochimica et Cosmochimica Acta 42.

Denlinger, D. L. 1971. Embryonic determination of pupal diapause in the flesh fly *Sarcophaga crassipalpis*. Journal of Insect Physiology 17:1815-1822.

Denlinger, D. L. 1972. Induction and termination of pupal diapause in *Sarcophaga* (Diptera: Sarcophagidae). Biological Bulletin 142:11-24.

Denlinger, D. L. 1981. Hormonal and metabolic aspects of pupal diapause in Diptera. Entomologia Generalis 7:245-259.

Denlinger, D. L. 2002. Regulation of Diapause. Annual Review of Entomology 47:93-122.

Denlinger, D. L., J. H. Willis, and G. Fraenkel. 1972. Rates and cycles of oxygen consumption during pupal diapause in *Sarcophaga* flesh flies. Journal of Insect Physiology 18:871-882.

Denver, R. J. 1997. Environmental stress as a developmental cue: corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. Hormones and Behavior 31:169-179.

Digby, L. 1995. Infant care, infanticide, and female reproductive strategies in polygynous groups of common marmosets (*Callithrix jacchus*). Behavioral Ecology and Sociobiology 37:51-61.

Doi, H., E. Kikuchi, S. Takagi, and S. Shikano. 2007. Changes in carbon and nitrogen stable isotopes of chironomid larvae during growth, starvation and metamorphosis. Rapid Communications in Mass Spectrometry 21:997-1002.

Ellers, J., and v. A. J. J. M. 1997. Life history evolution in *Asobara tabida*: plasticity in allocation of fat reserves to survival and reproduction. Journal of Evolutionary Biology 10:771-785.

Fischer, K., and K. Fiedler. 2001. Effects of adult feeding and temperature regime on fecundity and longevity in the butterfly *Lycaena hippothoe* (Lycaenidae). Journal of the Lepidopterist's Society 54:91-95.

Fischer, K., D. M. O'Brien, and C. L. Boggs. 2004. Allocation of larval and adult resources to reproduction in a fruit-feeding butterfly. Functional Ecology 18:656-663.

Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226:497-509.

Fraenkel, G., and C. Hsiao. 1968. Manifestations of a pupal diapause in two species of flies *Sarcophaga argyostoma* and *S. bullata*. Journal of Insect Physiology 14:689-705.

Gannes, L. Z., D. M. O'Brien, and C. Martinez del Rio. 1997. Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. Ecology 78:1271-1276.

Ghalambor, C. K., J. K. McKay, S. P. Carroll, and D. N. Reznick. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Functional Ecology 21:394-407.

Giron, D., and J. Casas. 2003. Mothers reduce egg provisioning with age. Ecology Letters 6:273-277.

Godfray, H. C. J., L. Partridge, and P. H. Harvey. 1991. Clutch size. Annual Review of Ecology and Systematics 22:409-429.

Good, T. P., and M. Tatar. 2001. Age-specific mortality and reproduction respond to adult dietary restriction in *Drosophila melanogaster*. Journal of Insect Physiology 47:1467-1473.

Goto, M., M. Fujii, K. Suzuki, and M. Sakai. 1998. Factors affecting carbohydrate and free amino acid content in overwintering larvae of *Enosima leucotaeniella*. Journal of Insect Physiology 44:87-94.

Gould, F., N. Blair, M. Reid, T. L. Rennie, J. Lopez, and S. Micinski. 2002. *Bacillus thuringiensis*-toxin resistance management: Stable isotope assessment of alternate host use by *Helicoverpa zea*. Proceedings of the National Academy of Sciences 99:16581-16586.

Hahn, D. A., and D. L. Denlinger. 2007. Meeting the energetic demands of insect diapause: nutrient storage and utilization. Journal of Insect Physiology 53:760-773.

Hahn, D. A., L. N. James, K. R. Milne, and J. D. Hatle. 2008. Life-history plasticity after attaining a dietary threshold for reproduction is associated with protein storage in flesh flies. Functional Ecology 22:1081-1090.

Hahn, D. A., M. N. Rourke, and K. R. Milne. 2008. Mating affects reproductive investment into eggs, but not the timing of oogenesis in the flesh fly *Sarcophaga crassipalpis*. Journal of Comparative Physiology B 178:225-233.

Han, E. N., and E. Bauce. 1998. Timing of diapause initiation, metabolic changes and overwintering survival of the spruce budworm, *Choristoneura fumiferana*. Ecological Entomology 23:160-167.

Harvey, W. R. 1962. Metabolic aspects of insect diapause. Annual Review of Entomology 7:57-88.

Hatle, J. D., D. W. Borst, M. E. Eskew, and S. A. Juliano. 2001. Maximum titers of vitellogenin and total hemolymph protein occur during the canalized phase of grasshopper egg production. Physiological and Biochemical Zoology 74:885-893.

Hatle, J. D., D. W. Borst, and S. A. Juliano. 2004. Plasticity and canalization in the control of reproduction in the lubber grasshopper. Integrative and Comparative Biology 43:635-645.

Hatle, J. D., S. A. Juliano, and D. W. Borst. 2000. Juvenile hormone is a marker of the onset of reproductive canalization in lubber grasshoppers. Insect Biochemistry and Molecular Biology 30:821-827.

Henn, M. R., and I. H. Chapela. 2000. Differential C isotope discrimination by fungi during decomposition of C_3- and C_4- derived sucrose. Applied and Environmental Microbiology 66:4180-4186.

Hilderbrand, G. V., S. D. Farley, C. T. Robbins, T. A. Hanley, K. Titus, and C. Servheen. 1996. Use of stable isotopes to determine diets of living and extinct bears. Canadian Journal of Zoology 74:2080-2088.

Hobbie, E. A., S. A. Macko, and H. H. Shugart. 1999. Insights into nitrogen and carbon dynamics of ectomycorrhizal and saprotrophic fungi from isotopic evidence. Oecologia 118:353-360.

Hobbie, E. A., N. S. Weber, and J. M. Trappe. 2001. Mycorrhizal vs saprotrophic status of fungi: the isotopic evidence. New Phytologist 150.

Hobson, K. A., and R. G. Clark. 1992. Assessing avian diets using stable isotopes I: turnover of ^{13}C in tissues. The Condor 94:181-188.

Hobson, K. A., D. M. Schell, D. Renouf, and E. Noseworthy. 1996. Stable carbon and nitrogen isotopic fractionation between diet and tissues of captive seals: implications for dietary reconstructions involving marine mammals. Canadian Journal of Fisheries and Aquatic Science 53:528-533.

Hogberg, P., A. H. Plamboeck, A. F. S. Taylor, and P. M. A. Fransson. 1999. Natural ^{13}C abundance reveals trophic status of fungi and host-origin of carbon in mycorrhizal fungi in mixed forests. Proceedings of the National Academy of Sciences 96:8534-8539.

Hood-Nowotny, R., and B. G. J. Knols. 2007. Stable isotope methods in biological and ecological studies of arthropods. Entomologia Experimentalis et Applicata 124:3-16.

Huck, U., R. L. Soltis, and C. B. Coopersmith. 1982. Infanticide in male laboratory mice: effects of social status, prior sexual experience, and basis for discrimination between related and unrelated young. Animal Behaviour 30:1158-1165.

Hutchings, J. A. 1991. Fitness consequences of variation in egg size and food abundance in brook trout *Salvelinus fontinalis*. Evolution 45:1162-1168.

Ishihara, M., and M. Shimada. 1995. Trade-off in allocation of metabolic reserves - effects of diapause on egg production and adult longevity in a multivoltine Bruchid, *Kytorhinus sharpianus*. Functional Ecology 9:618-624.

Jervis, M. A., C. L. Boggs, and P. N. Ferns. 2005. Egg maturation strategy and its associated trade-offs: a synthesis focusing on Lepidoptera. Ecological Entomology 30:359-375.

Jervis, M. A., C. L. Boggs, and P. N. Ferns. 2007. Egg maturation strategy and survival trade-offs in holometabolous insects: a comparative approach. Biological Journal of the Linnaean Society 90:293-302.

Jervis, M. A., J. Ellers, and J. A. Harvey. 2008. Resource acquisition, allocation, and utilization in parasitoid reproductive strategies. Annual Review of Entomology 53:361-385.

Jonsson, K. I. 1997. Capital and income breeding as alternative tactics of resource use in reproduction. OIKOS 78:57-66.

Juliano, S. A., J. R. Olson, E. G. Murrell, and J. D. Hatle. 2004. Plasticity and canalization of insect reproduction: testing alternative models of life history transitions. Ecology 85:2986-2996.

Kaitala, A. 1988. Wing muscle dimorphism: two reproductive pathways of the waterstrider *Gerris thoracicus* in relation to habitat instability. OIKOS 53:222-228.

Karasov, W. H., and C. Martinez del Rio. 2007. Isotopic Ecology, Physiological Ecology. Princeton, Princeton University Press.

Kohzu, A., T. Yoshioka, T. Ando, M. Takahashi, K. Koba, and E. Wada. 1999. Natural ^{13}C and ^{15}N abundance of field-collected fungi and their ecological implications. New Phytologist 144:323-330.

Kono, Y. 1970. Photoperiodic induction of diapause in *Pieris rapae crucivora* Boisduval (Lepidoptera:Pieridae). Applied Entomology and Zoology 5:213-224.

Lee, R. E., C. P. Chen, M. H. Meacham, and D. L. Denlinger. 1987. Ontogenetic patterns of cold-hardiness and glycerol production in *Sarcophaga crassipalpis*. Journal of Insect Physiology 33:587-592.

Lefevere, K. S., A. B. Koopmanscap, and C. A. D. De Kort. 1989. Changes in the concentrations of metabolites in haemolymph during and after diapause in female Colorado potato beetle, *Leptinotarsa decemlineata*. Journal of Insect Physiology 35:121-128.

Lighton, J. R. B. 2008, Measuring metabolic rates: a manual for scientists. New York, Oxford University Press.

Logan, J. M., T. D. Jardine, T. J. Miller, S. E. Bunn, R. A. Cunjak, and M. E. Lutcavage. 2008. Lipid corrections in carbon and nitrogen stable isotope analyses: comparison of chemical extraction and modeling methods. Journal of Animal Ecology 77:838-846.

MacFadden, B. J., T. E. Cerling, J. M. Harris, and J. Prado. 1999. Ancient latitudinal gradients of C_3/C_4 grasses interpreted from stable isotopes of new world Pleistocene horse (*Equus*) teeth. Global Ecology and Biogeography 8:137-149.

Madigan, M. T., J. M. Martinko, and J. Parker. 2003, Biology of Microorganisms. Upper Saddle River, Prentice Hall.

Magnhagen, C. 1991. Predation risk as a cost of reproduction. TRENDS in Ecology and Evolution 6:183-186.

Martinez del Rio, C., and B. O. Wolf. 2005. Mass-balance models for animal isotopic ecology in J. M. Stark, and T. Wang, eds. Physiological and Ecological Adaptations to Feeding in Vertebrates. Enfield, NH, Science Publishers Inc.

Martinez del Rio, C., N. Wolf, S. A. Carleton, and L. Z. Gannes. 2009. Isotopic ecology ten years after a call for more laboratory experiments. Biological Reviews 84:91-111.

Matsuo, Y. 2006. Cost of prolonged diapause and its relationship to body size in a seed predator. Functional Ecology 20:300-306.

McCutchan, J. H., W. M. Lewis, C. Kendall, and C. C. McGrath. 2003. Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. OIKOS 102:378-390.

Melzer, E., and H. L. Schmidt. 1987. Carbon isotope effects on the pyruvate dehydrogenase reaction and their importance for relative carbon-13 depletion in lipids. The Journal of Biological Chemistry 262:8159-8164.

Min, K. J., M. F. Hogan, M. Tatar, and D. M. O'Brien. 2006. Resource allocation to reproduction and soma in *Drosophila*: a stable isotope analysis of carbon from dietary sugar. Journal of Insect Physiology 52:763-770.

Moehrlin, G. S., and S. A. Juliano. 1998. Plasticity of insect reproduction: testing models of flexible and fixed development in response to different growth rates. Oecologia 115:492-500.

Moore, P. J., and A. J. Moore. 2001. Reproductive aging and mating: the ticking of the biological clock in female cockroaches. . Proceedings of the National Academy of Sciences 98:9171-9176.

Moore, P. J., and S. Sharma. 2005. A delay in age at first mating results in the loss of future reproductive potential via apoptosis. Evolution and Development 7:216-222.

Morgan, T. D., and G. M. Chippendale. 1983. Free amino acids of the haemolymph of the southwestern corn borer and the European corn borer in relation to their diapause. Journal of Insect Physiology 29:735-740.

Nijhout, H. F. 1994, Insect Hormones. Princeton, Princeton University Press.

Noordahl, K., and E. Korpimaki. 1995. Does predation risk constrain maturation in cyclic vole populations? OIKOS 72:263-272.

O'Brien, D. M., C. L. Boggs, and M. L. Fogel. 2004. Making eggs from nectar: the role of life history and dietary carbon turnover in butterfly reproductive resource allocation. OIKOS 105:279-291.

O'Brien, D. M., C. L. Boggs, and M. L. Fogel. 2005. The amino acids used in reproduction by butterflies: a comparative study of dietary sources using compound-specific stable isotope analysis. Physiological and Biochemical Zoology 78:819-827.

O'Brien, D. M., M. L. Fogel, and C. L. Boggs. 2002. Renewable and nonrenewable resources: amino acid turnover and allocation to reproduction in Lepidoptera. Proceedings of the National Academy of Sciences 99:4413-4418.

O'Brien, D. M., D. P. Schrag, and C. Martinez del Rio. 2000. Allocation to reproduction in a hawkmoth: a quantitative analysis using stable carbon isotopes. Ecology 81:2822-2831.

Packer, C., and A. E. Pusey. 1983. Adaptations of female lions to infanticide by incoming males. The American Naturalist 121:716-728.

Podlesak, D. W., and S. R. McWilliams. 2007. Metabolic routing of dietary nutrients in birds: effects of dietary lipid concentration on $\delta^{13}C$ of depot fat and its ecological implications. The Auk 124:916-925.

Ponsard, S., M. T. Bethenod, A. Bontemps, L. Pelozuelo, M. C. Souqual, and B. D. 2004. Carbon stable isotopes: a tool for studying the mating, oviposition, and spatial distribution of races of European corn borer, *Ostrina nubilalis*, among host plants in the field. Canadian Journal of Zoology 82:1177-1185.

Post, D. M., C. A. Layman, D. A. Arrington, G. Takimoto, J. Quattrochi, and C. G. Montana. 2007. Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. Oecologia 152:179-189.

Ragland, G. J., J. Fuller, J. L. Feder, and D. A. Hahn. 2009. Biphasic metabolic rate trajectory of pupal diapause termination and post-diapause development in a tephritid fly. Journal of Insect Physiology 55:344-350.

Reznick, D. N. 1985. Costs of reproduction: an evaluation of the empirical evidence. OIKOS 44:257-267.

Reznick, D. N. 1990. Plasticity in age and size at maturity in male guppies (*Poecilia reticulata*): An experimental evaluation of alternative models of development. Journal of Evolutionary Biology 3:185-203.

Reznick, D. N., L. Nunney, and A. Tessier. 2000. Big houses, big cars, superfleas and the costs of reproduction. TRENDS in Ecology and Evolution 15:421-425.

Reznick, D. N., and A. P. Yang. 1993. The influence of fluctuating resources on life history: patterns of allocation and plasticity in female guppies. Ecology 74:2011-2019.

Rickards, J., M. J. Kelleher, and K. B. Storey. 1987. Strategies of freeze avoidance in larvae of the goldenrod gall moth, *Epiblema scudderiana*: winter profiles of a natural population. Journal of Insect Physiology 33:443-450.

Ricklefs, R. E., and M. Wikelski. 2002. The physiology/ life-history nexus. TRENDS in Ecology and Evolution 17:462-468.

Rinehart, J. P., and D. L. Denlinger. 2000. Heat-shock protein 90 is down-regulated during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. Insect Molecular Biology 9:641-645.

Rivero, A., and J. Casas. 1999. Incorporating physiology into parasitioid behavioral ecology: the allocation of nutritional resources. Researches on Population Ecology 41:39-45.

Rivero, A., D. Giron, and J. Casas. 2001. Lifetime allocation of juvenile and adult nutritional resources to egg production in a holometabolous insect. Proceedings of the Royal Society of London B 268:1231-1237.

Rose, F. L., T. R. Simpson, and R. W. Manning. 1996. Measured and predicted egg volume of *Pseudemys texana* with comments on turtle egg shape. Journal of Herpetology 30:433-435.

Rossmann, A., M. Butzenlechner, and H. L. Schmidt. 1990. Evidence for a nonstatistical carbon isotope distribution in natural glucose. Plant Physiology 96:609-614.

Ruess, L., A. Tiunov, D. Haubert, H. H. Richnow, M. M. Haggblom, and S. Scheu. 2005. Carbon stable isotope fractionation and trophic transfer of fatty acids in fungal based soil food chains. Soil Biology and Biochemistry 37:945-953.

Saunders, D. S. 1997. Under-sized larvae from short-day adults of the blowfly *Calliphora vicina*, side-step the diapause programme. Physiological Entomology 22:249-255.

Saunders, D. S. 2000. Larval diapause duration and fat metabolism in three geographical strains of the blowfly, *Calliphora vicina*. Journal of Insect Physiology 46:509-517.

Seigert, K. J. 1986. The effects of chilling and integumentary injury on carbohydrate and lipid metabolism in diapause and non-diapause pupae of *Manduca sexta*. Comparative Biochemistry and Physiology 85A:257-262.

Shine, R., and G. P. Brown. 2008. Adapting to the unpredictable: reproductive biology of vertebrates in the Australian wet-dry tropics. Philosophical Transactions of the Royal Society B 363:363-373.

Sibly, R. M., and P. Calow. 1984. Direct and absorption costing in the evolution of life cycles. Journal of Theoretical Biology 111:463-473.

Somme, L. 1982. Supercooling and winter survival in terrestrial arthropods. Comparative Biochemistry and Physiology 73A:519-543.

Spitze, K. 1991. *Chaoborus* predation and life-history evolution in *Daphnia pulex*: temporal pattern of population diversity, fitness, and mean life history. Evolution 45:82-89.

Spitze, K., J. Burnson, and M. Lynch. 1991. The covariance structure of life-history characters in *Daphnia pulex*. Evolution 45:1081-1090.

Stearns, S. C. 1992, The evolution of life histories. New York, Oxford University Press.

Stephens, P. A., I. L. Boyd, J. M. McNamara, and A. I. Houston. 2009. Capital breeding and income breeding: their meaning, measurement, and worth. Ecology 90:2057-2067.

Storey, K. B. 1997. Organic solutes in freezing tolerance. Comparative Biochemistry and Physiology 117A:319-326.

Tatar, M., and J. R. Carey. 1995. Nutrition mediates reproductive trade-offs with age-specific mortality in the beetle *Callosobruchus maculatus*. Ecology 76:2066-2073.

Tayasu, I., T. Abe, P. Eggleton, and D. E. Bignell. 1997. Nitrogen and carbon isotope ratios in termites: an indicator of trophic habit along the gradient from wood-feeding to soil-feeding. Ecological Entomology 22:343-351.

Terri, J. A., and L. G. Stowe. 1976. Climactic patterns and the distribution of C_4 grasses in North America. Oecologia 23:1-12.

Tibbets, T. M., L. A. Wheeless, and C. Martinez del Rio. 2008. Isotopic enrichment without change in diet: an ontogenetic shift in $\delta^{15}N$ during insect metamorphosis. Functional Ecology 22:109-113.

Tsumuki, H., R. R. Rojas, K. B. Storey, and J. G. Baust. 1987. The fate of [^{14}C] glucose during cold-hardening in *Eurosta solidaginis* (Fitch). Insect Biochemistry and Molecular Biology 17:347-352.

Twombly, S. 1996. Timing of metamorphosis in a freshwater crustacean: comparison with anuran models. Ecology 77:1855-1866.

Valder, S. M., T. L. Hopkins, and S. A. Valder. 1969. Diapause induction and changes in lipid composition in diapausing and reproducing faceflies, *Musca autumnalis*. Journal of Insect Physiology 15:1199-1214.

van Handel, E. 1965. Microseparation of glycogen, sugars, and lipids. Analytical Biochemistry 11:266-271.

van Handel, E. 1985. Rapid determination of glycogen and sugars in mosquitoes. Journal of the American Mosquito Association 1:299-301.

van Noordwijk, A. J., and G. de Jong. 1986. Acquisition and allocation of resources: their influence on variation in life history tactics. The American Naturalist 128:137-142.

Vanderklift, M. A., and S. Ponsard. 2003. Sources of variation in consumer-diet δ^{15}N enrichment: a meta-analysis. Oecologia 136:169-182.

Varpe, O., C. Jorgensen, G. A. Tarling, and O. Fiksen. 2009. The adaptive value of energy storage and capital breeding in seasonal environments. OIKOS 118:363-370.

Voet, D., and J. G. Voet. 2004, Biochemistry: Biomolecules, Mechanisms of Enzyme Action, and Metabolism, v. 1. Hoboken, John Wiley & Sons, Inc.

Warbrick-Smith, J., S. T. Behmer, K. P. Lee, D. Raubenheimer, and S. J. Simpson. 2006. Evolving resistance to obesity in an insect. Proceedings of the National Academy of Sciences 103:14045-14049.

Warner, D. A., X. Bonnet, K. A. Hobson, and R. Shine. 2008. Lizards combine stored energy and recently acquired nutrients flexibility to fuel reproduction. Journal of Animal Ecology 77.

Welch, K. C., L. G. Herrera, and R. K. Suarez. 2008. Dietary sugar as a direct fuel for flight in the nectarivorous bat *Glossophaga soricina*. The Journal of Experimental Biology 211:310-316.

Welch, K. C., and R. K. Suarez. 2007. Oxidation rate and turnover of ingested sugar in hovering Anna's (*Calypte anna*) and rufous (*Selasphorus rufus*) hummingbirds. The Journal of Experimental Biology 210:2154-2162.

Wessels, F. J., and D. A. Hahn. 2010. Carbon 13 discrimination during lipid biosynthesis varies with dietary concentration of stable isotopes: implications for stable isotope analyses. Functional Ecology.

Wessels, F. J., D. C. Jordan, and D. A. Hahn. 2010a. Allocation from capital and income sources to reproduction shift from first to second clutch in the flesh fly, *Sarcophaga crassipalpis*. Journal of Insect Physiology.

Wessels, F. J., R. Kristal, and F. Netter. 2010b. Does it pay to delay? Flesh flies show adaptive plasticity in reproductive timing. Oecologia: In Press.

West-Eberhard, M. J. 1989. Phenotypic plasticity and the origins of diversity. Annual Review of Ecology and Systematics 20:249-278.

Wheeler, D. 1996. The role of nourishment in oogenesis. Annual Review of Entomology 41:407-431.

Wilbur, H. M., and J. P. Collins. 1973. Ecological aspects of amphibian metamorphosis. Science 182:1305-1314.

Williams, J. B., J. D. Shorthouse, and R. E. Lee. 2003. Deleterious effects of mild simulated overwintering temperatures on survival and potential fecundity of rose-galling *Diplolepis* wasps (Hymenoptera: Cynipidae). Journal of Experimental Zoology 298A:23-31.

Wingfield, J. C. 2005. Flexibility in annual cycles of birds: implications for endocrine control mechanisms. Journal of Ornithology 146:291-304.

Wipking, W., M. Viebahn, and D. Neumann. 1995. Oxygen consumption, water, lipid and glycogen content of early and late diapause and non-diapause larvae of the burnet moth, *Zygaena trifolii*. Journal of Insect Physiology 41:47-56.

Wolff, J. O. 1997. Population regulation in mammals: an evolutionary perspective. Journal of Animal Ecology 66:1-13.

Woodroffe, R., and D. W. MacDonald. 1995. Female/female competition in European badgers *Meles meles*: effects on breeding success. Journal of Animal Ecology 64:12-20.

Wyatt, G. R. 1967. The biochemistry of sugars and polysaccharides in insects. Advances in Insect Physiology 4:287-360.

Ylonen, H. 1989. Weasels *Mustela nivalis* supress reproduction in cyclic bank voles *Clethrionomys glareolus*. OIKOS 55:138-140.

Yocum, G. D., W. P. Kemp, J. Bosch, and J. N. Knoblett. 2005. Temporal variation in overwintering gene expression and respiration in the solitary bee *Megachile rotundata*. Journal of Insect Physiology 51:621-629.

Zera, A. J. 2005. Intermediary metabolism and life history trade-offs: lipid metabolism in lines of the wing-polymorphic cricket, *Gryllus firmus*, selected for flight capability vs. early age reproduction. Integrative and Comparative Biology 45:511-524.

Zera, A. J., and L. G. Harshman. 2001. The physiology of life history trade-offs in animals. Annual Review of Ecology and Systematics 32:95-126.

Zera, A. J., S. Mole, and K. Rokke. 1994. Lipid, carbohydrate and nitrogen content of long- and short-winged *Gryllus firmus*: implications for the physiological cost of flight capability. Journal of Insect Physiology 40:1037-1044.

Zera, A. J., J. Potts, and K. Kobus. 1998. The physiology of life-history trade-offs: experimental analysis of a hormonally induced life-history trade-off in *Gryllus assimilis*. The American Naturalist 152:7-23.

Zera, A. J., and Z. Zhao. 2006. Intermediary metabolism and life-history trade-offs: differential metabolism of amino acids underlies the dispersal-reproduction trade-off in a wing-polymorphic cricket. The American Naturalist 167:889-900.

Zhao, Z., and A. J. Zera. 2002. Differential lipid biosynthesis underlies a tradeoff between reproduction and flight capability in a wing-polymorphic cricket. Proceedings of the National Academy of Sciences 99:16829-16834.

Zhou, G., M. Flowers, K. Friedrich, J. Horton, J. E. Pennington, and M. A. Wells. 2004. Metabolic fate of [^{14}C]-labeled meal protein amino acids in *Aedes aegypti* mosquitoes. Journal of Insect Physiology 50:337-349.

Zhou, G., and R. L. Miesfeld. 2009. Energy metabolism during diapause in *Culex pipiens* mosquitoes. Journal of Insect Physiology 55:40-46.

BIOGRAPHICAL SKETCH

Frank Wessels was born in 1980 in Indianapolis, IN and moved to Minnesota before finally settling in Columbus, OH, where he grew up. From a young age Frank showed an interest in biology, exploring the creeks and wooded areas surrounding his house. This curiosity ultimately led Frank to the University of Tampa, where he received a Bachelor of Science in marine science and biology in 2002. Internships at the Department of Environmental Protection and Dow AgroSciences piqued his interest in entomology, leading Frank to complete his Master of Science in entomology at the University of Florida in 2005. After a short break working on termite baiting for Dow AgroSciences as a consultant, Frank entered his Doctor of Philosophy program at his Alma Mater, the University of Florida in 2006.