THE ROLE OF CONTEXT IN INVESTMENT INTO REPRODUCTIVE TISSUE AND IMPLICATIONS FOR MATING

by

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DEDICATION

I dedicate this dissertation to my parents, Dennis and Susan Carsten. The example of love, dedication to family, and hard work that they set has strongly influenced me throughout my life. I thank them for instilling me with a love of learning and a curiosity about nature, and most of all, for encouraging me to be my own person.

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ABSTRACT

Reproductive traits are often thought of as fixed, genetically determined properties. However, such traits are often dynamic, exhibiting different expression patterns depending on context. Both internal state and external environment can have a strong effect on how traits are expressed. Variation in these factors across the lifetime of an individual should select for flexibility in trait expression, rather than fixation.

My dissertation work examines how mating behavior and testes size respond to several previously unexplored contextual factors, using *Rhagoletis juglandis*, the walnut fly, as a model system. For mating behavior, I predicted that differences in female reproductive state (egg load) and experience with host resource would impact mating decisions. For testes size, I predicted that social environment (sex ratio) and changes in resource environment would determine testes size.

Behavioral observations of flies showed that a large egg load increased the likelihood of copulation, while prior experience with host fruit decreased copulation time. These results are the first to distinguish effects of experience on physiological state from other effects of experience in the context of mating behavior.

Manipulation of the sex ratio revealed that males develop larger testes when reared in an environment with many potential competitors. This is the first study to show that that allocation to a male reproductive organ can change depending on the sex ratio. My studies showed that resource environment is also important in determining testes investment patterns. When adult males are deprived of protein, they develop smaller testes. A stable isotope analysis of testes further confirms that resource environment is

important for testes development. Males rely more on nitrogen derived at the larval stage than at the adult stage, but adult carbon sources are a large component of testes mass.

In sum, this dissertation demonstrates the importance of context in the expression of reproductive traits. Recent research has shown that such traits can respond more dynamically to context than previously thought, but this area of research is young. My results help provide a greater understanding of the processes shaping the evolution of reproductive traits.

I. INTRODUCTION

Explanation of the problem

Reproductive traits, both physiological and behavioral, have important fitness consequences for both males and females, and thus are subject to strong selection. Strong selection often drives rapid, unidirectional change in traits; thus, the genetic variance in such traits in a given population should be small. For instance, if large ejaculates predict paternity success in a particular environment, then males with smaller ejaculates will leave fewer offspring, quickly reducing variance in ejaculate size in that population. The argument for rapid selection and fixation of traits assumes that environment is a constant for individuals in a population. However, the experienced environment can vary between individuals or across the lifetime of individuals in the same population. These different environmental contexts (factors both internal and external to the animal) can have profound effects on both the strength and direction of selection. Where the context of reproductive opportunity differs, there may be selection for flexibility (i.e., plasticity) in reproductive traits, rather than a drive to fixation.

A number of studies have shown flexibility in reproductive traits in response to a wide variety of contextual factors (e.g. Hooper et al. 1999, Engqvist and Sauer 2002, Badyaev and Qvarnstrom 2002). For example, Hebets (2003) showed that among wolf spiders, juvenile exposure to a specific male phenotype created an adult mating preference for that same phenotype. However, few studies have examined how other biologically important contexts, such as female investment in reproductive tissue, affect mating decisions. Similarly, several studies have shown that ejaculate size varies in

response to the presence of rival males (rev. in Wedell et al. 2002). But despite the underlying importance of testes size in ejaculate production, there has been almost no investigation into how investment in testes might vary in response to contextual factors.

This dissertation investigates the relationship between environmental context and investment into female and male reproductive tissue, using *Rhagoletis juglandis*, the walnut husk fly, as a model system. In this species, ovarian investment is tightly linked to resource environment. This work examines how mating decisions are determined by variation in egg load and resource environment. I also ask how allocation to testes is affected by resource and social environments that may predict mating opportunities.

Ovarian dynamics and mating

Mating decisions can be shaped by a number of selection pressures. Males are generally selected to maximize mating opportunities, as fecundity increases with increasing mate number (Bateman 1948). However, males may decrease effort towards maximizing mate number, and focus on monopolizing fewer mates, depending on the mating context. In particular, female reproductive state represents an important factor for male mating decisions. Females in good reproductive condition may be more fecund than other females; thus, a male might prefer to copulate with more fecund females (Danielson-Francois et al., 2002, Kelso and Verrell, 2002). He might also choose to spend time guarding a more fecund female to prevent her from mating with other males. If a male does not have the opportunity to mate with few, highly fecund females, his time might be better spent in pursuing mating opportunities with many, less fecund females.

However, a mating event represents the balance of female and male decisions. The selection on female mating decisions could arise from a variety of sources, but the pressure to provide optimal parental care may often be a key factor that determines how mating decisions are made. For insects, parental care is often limited to oviposition; thus, the quality and availability of a reproductive resource is a critical factor in shaping oviposition decisions, and ultimately, mating decisions. Among walnut flies, oviposition opportunities are spatially and temporally limited. Walnut trees in southern Arizona typically occur in clumps that are spatially dispersed from each other. Because of the long distances between trees or clumps of trees, it is believed that female dispersion from the pupal emergence site is limited (Nufio et al. 2000). There are time constraints on oviposition opportunities, as well. Within each area, all walnut fruit is generally used within 2 ½ weeks (Nufio et al. 2000). These spatial and temporal limits on the resource have important consequences for reproductive biology; females have a limited time budget in which to both mate and lay eggs. There are reasons for females to allocate more time to laying eggs than to mating. Females can develop multiple clutches within a season; thus, the less time a female spends in mating activities, the more time she has to develop and lay more eggs, which will maximize her fecundity. Thus, if there is high quality fruit available, females should tend to copulate for shorter durations (given that females have some control over copulation duration), as long copulations could be costly in terms of reducing time available for laying eggs (Sherman, 1983).

There are potential costs to short copulations, however. For instance, short copulations might result in higher levels of male harassment. Male density on and around

the fruit can be high, and females arriving at a given fruit often mate multiply in what appear to be "forced" copulations (Nufio et al. 2000). A female that engages in a long copulation with a single male may be protected from this type of harassment. This may lead to an increase in time available to lay eggs, as the recently mated male often hovers near the female during subsequent egg laying, allowing her to lay eggs without interruption from other males. While she has "lost" time to lay eggs by engaging in a long copulation with one male, this still represents an overall time savings, as she will not be engaging in multiple matings with many different males prior to laying eggs. Thus, if no high quality fruit are readily available, a female may choose to engage in a longer copulation. These scenarios illustrate the importance of context in shaping mating decisions. It would be beneficial to both males and females to retain a degree of flexibility in mating decisions, as different reproductive and resource contexts might dictate different paths to the maximization of fecundity.

Allocation to testes

Testis size is an important reproductive trait that is primarily influenced by the intensity of sperm competition. Theory predicts that males should invest more in ejaculates where the risk of sperm competition is high (Parker 1998). Because larger testes generally produce more sperm (Schärer *et al*. 2004), testes size should increase when the potential for sperm competition is high. A number of studies have shown that relative testes size increases across taxa as sperm competition risk increases (Svärd & Wiklund 1989; Gage

1994; Stockley *et al*. 1997; Pitcher *et al*. 2005). However, the idea that testes size might vary intraspecifically in response to specific contexts has been much less explored.

The social environment that an individual experiences can provide a measure of the intensity of sperm competition likely to be encountered (Shuster and Wade 2003). In particular, the operational sex ratio (the number of sexually active males in a population relative to the number of receptive females; Emlen & Oring 1977) determines the number of potential competitors in the local environment. When the OSR is male-biased, an increase in testes size should be beneficial to a male, allowing him to numerically outcompete rivals during sperm competition (Birkhead and Moller 1998). While maximal investment into testes may benefit a male under the context of a male-biased sex ratio, it might better serve a male to invest his limited resources in different activities when the sex ratio is female-biased. In this context, males will experience little direct competition within the female reproductive tract. While producing enough sperm to fertilize many eggs is still important, producing the voluminous amounts of sperm necessary to outcompete a rival is less important. Males may elect to economize testes size, parceling sperm out among many females and allowing greater energetic allocation to activities such as pursuing many mates. Such conflicting selection pressures may select for flexibility in testes size in response to context, rather than fixed testes size.

The arguments presented above for selection favoring flexibility in traits rely on the assumption that potentially beneficial activities must compete for limited resources. A physiologically-based understanding of the source of nutrients allocated to specific functions, as well as the consequences of any shortages, can help refine this assumption

by defining which activities might directly compete for resources. Understanding from where nutrients derive is especially important for animals that have spatially or temporally separate resource pools across their lifetimes, such as holometabolous insects. The resources available to insects can be thought of as deriving from two separate pools, one acquired at the larval stage, and one acquired at the adult stage (Boggs 1981; Zera and Harshman 2001). Depending on how these resources are allocated to different functions, allocation to testes may or may not compete with other activities that influence reproductive success. For instance, if resources for testes derive mostly from larval sources, and resources for courtship display derive mostly from adult sources, then these activities will not be forced to trade off. However, if resources for these functions are derived from the same pool, then shortages in that pool will generate the need for a tradeoff. In short, the resource pool could affect how selection acts on these traits.

Explanation of dissertation format

The dissertation research presented here examines flexibility in mating traits and testes allocation, as well as the source of nutrients allocated to testes, and the consequences of nutrient shortages at the adult stage. All five appendices represent work that I conducted and papers that I produced. For appendices A, B, and C, Dan Papaj's co-authorship represents his substantial intellectual contribution to the projects and the papers. For appendix C, Diane O'Brien's co-authorship represents her substantial intellectual contribution to the project and the paper. Appendix D represents a project that I produced primarily on my own, while Appendix E represents supplemental data.

Appendix A, "Effects of reproductive state and host resource experience on mating decisions in a walnut fly," examines how mating decisions are affected by female reproductive state and prior experience with walnut fruit. My results show that each context affects mating behavior, but in diverse ways. This is the first study to distinguish effects of experience on physiological state from other effects of experience in the context of mating behavior.

Appendix B, "Sex ratio during development determines testes size in a tephritid fly," examines the response of testes size to an important social context, operational sex ratio. This is the first study to show that allocation to a male reproductive organ can change depending on the operational sex ratio. The results also show that, while flexible responses to context are generally fast, they can also act on a relatively slow time scale.

Appendix C, "Resource allocation to testes in walnut flies: diverse strategies for carbon and nitrogen" looks at relative allocation to testes from larval vs. adult resources.

The results demonstrate that walnut flies use mostly larval nitrogen in testes, but that adult carbon is an important constituent of testes. This study is one of the first to demonstrate that animals can use distinct allocation strategies for different nutrients, rather than a single overall allocation strategy.

Appendix D, “Role of adult protein source in mating effort and testes size,” examines how testes size and mating effort respond to protein restriction at the adult stage. The results suggest that allocation of resources to testes and mating effort compete within the organism.

Appendix E constitutes supplemental data that I collected, which helps support some of the inferences in other studies. While not a large enough data set to constitute a full chapter, these data nonetheless are important to include in the dissertation.

II. PRESENT STUDY

The methods, results, and conclusions of this study are presented in the paper appended to this dissertation/thesis. The following is a summary of the most important findings in this document.

Appendix A. Prior experience with conspecifics or essential resources, as well as physiological condition, can have important influences on an animal's reproductive behavior. While effects of experience and physiological state are generally treated separately, they often interact. In this study, we found that females with large egg loads were more likely to copulate than those with small egg loads, and that prior experience with fruit resulted in decreases in copulation time. This is the first study to distinguish effects of experience on physiological state from other effects of experience in the context of mating behavior.

Appendix B. Traits such as testes size are thought to experience strong selection due to sperm competition. At the interspecific level, species that experience strong sperm competition generally have evolved larger testes than those experiencing weak sperm competition. However, levels of sperm competition may vary in time in space, leading to selection for flexibility in testes size within a species. Intraspecific patterns of sperm competition are seldom explored. In this study, we found for the first time that operational sex ratio, which predicts the level of sperm competition an individual is likely to experience, influences testes size: males develop larger testes in more male-biased sex ratios compared to more female-biased sex ratios.

Appendix C. Testes size often predicts whether a male will win during episodes of sperm competition. However, little is known about the source of nutrients allocated to testes development. Among holometabolous insects, metabolic resources can be derived from the larval or the adult diet. Distinguishing the source of nutrients allocated to testes can shed light on life history factors that shape the evolution of male reproductive strategies. We find that, instead of possessing a single allocation strategy, walnut flies are capital breeders for nitrogen (relying on larval reserves), and income breeders for carbon (relying more on adult resources). Nitrogen is critical for biosynthesis of tissue; thus, the larval environment is critically important for the development of testes and ultimately, the sperm competitive ability of males.

Appendix D. Sexual selection can act on males both before and after copulation, favoring different suites of reproductive traits. Although it would be beneficial for a male to invest maximally in traits that help him compete both before and after copulation, limitations in available resources might dictate that investment into such traits trades off. I examined the relationship between mating success and investment into testes under conditions of unlimited access to protein and conditions of protein restriction in adult walnut flies, *Rhagoletis juglandis.* My results indicate that, when males have unlimited access to protein, they increase mating effort at the expense of testes size. Conversely, when males have no access to protein, they step up investment to testes but reduce mating effort. These results suggest that testes and mating effort may compete for resources within the organism.

Appendix E. Body size has important consequences for walnut fly reproductive strategy. Larger males often have an advantage in contests, which in turn can lead to increased mating opportunity. The supplemental data in Appendix E shows that large males have a mating advantage over small males in direct competition, as well. Large males are more likely to achieve the first copulation, as well as achieving more copulations overall, in comparison to small males.

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APPENDIX A

EFFECTS OF REPRODUCTIVE STATE AND HOST RESOURCE EXPERIENCE ON MATING DECSIONS IN A WALNUT FLY

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Title: Effects of reproductive state and host resource experience on mating decisions in a walnut fly

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Title: Effects of reproductive state and host resource experience on mating decisions in a walnut-infesting fly (*Rhagoletis juglandis*)

Running title: Experience and mating decisions in walnut-infesting flies

Abstract

Prior experience with conspecifics or essential resources, as well as physiological condition, can have important influences on an animal's reproductive behavior. While effects of experience and physiological state (such as reproductive condition) are generally treated separately in theoretical discussions, they often interact. No previous study has attempted to distinguish effects of experience on physiological state from other effects of experience in the context of mating behavior. In a study of a walnut-infesting tephritid fly (*Rhagoletis juglandis*), we examined the effects of host fruit experience on mating behavior. We manipulated physiological state in terms of egg load (defined as the number of mature oocytes in a female's ovaries) independently of fruit experience to distinguish the effects of these variables. We found that females with high egg loads were significantly more likely to copulate than low egg load females; level of fruit experience had no effect on propensity to copulate, except via effects on egg load. In contrast, females with prior exposure to fruit copulated for a significantly shorter duration than control females, while egg load had no effect on copulation duration. These results suggest that female reproductive condition and exposure to essential resources can have important, albeit diverse effects on mating behavior. We discuss how distinguishing

different types of variables may provide insight into sexual conflict over mating decisions, as well as which sex controls specific aspects of behavior.

Keywords: mating, prior experience, resource defense, copulation duration, egg load, sexual conflict

Introduction

Reproductive decisions such as mate choice, length of copulation, egg laying, and territorial defense have important fitness consequences for both males and females. Over the last two decades, it has been increasingly recognized that such decisions are shaped by a variety of social and environmental factors. In particular, experience with conspecifics has been shown to shape mating behavior across a number of taxa. For instance, Hebets (2003) showed that among wolf spiders, juvenile exposure to a specific male phenotype created an adult mating preference for that same phenotype. Similarly, Rosenqvist and Houde (1997) showed that exposure to males with different amounts of orange coloration influenced the mating preferences of female guppies.

In resource-based mating systems, experience with an essential resource, such as a host plant or territory, can also influence mating behavior. Male seaweed flies, for instance, are more likely to mount females if they have had prior exposure to seaweed (Dunn et al., 2002). Prokopy et al. (1989) showed that male apple maggot flies, which guard host fruit and mate with females as they arrive, increase their territorial behavior after gaining experience with the host fruit. Similarly, Hyman et al. (2004) showed that male song sparrows that previously held territories were more likely to engage in territorial defense than those that never held territories.

Authors of these studies frequently infer or imply that learning is the basis for effects of experience on behavior (Kodric-Brown and Nicoletto, 2001; Wagner et al., 2001; Slagsvold et al., 2002; White et al., 2002; Witte and Sawka, 2003). While such behavioral changes must sometimes reflect learning or imprinting, experience can also

change behavior by altering an animal's physiological (including reproductive) state. In *Drosophila* females, for example, mating induces rapid oogenesis as well as a host of behavioral changes, including reduced receptivity (reviewed in Chapman, 2001). In Caribbean fruit flies, mating experience elevates juvenile hormone titers in males, causing them to release pheromone at higher rates in which in turn leads to greater mating success (Teal et al., 2000). In piñon jays, experience with an abundance of piñon seeds leads to increased testis growth and earlier breeding (Ligon, 1978). In these examples, learning is unlikely to play a role in mediating these physiological changes and the resulting behavioral shifts; for instance, the changes in *Drosophila* behavior are directly mediated by male accessory gland products (Chapman, 2001).

Sometimes the underlying mechanisms mediating behavioral shifts can be unclear. Landolt (1994) showed that female papaya flies increased their remating frequency in the presence of host fruit; however, it is not clear whether this shift is mediated by learning or by some direct effect on physiological state. In another study, Grieco et al. (2002) showed that blue tits shifted their egg laying date in response to food availability in the previous year, and implies that this experiential change is a result of learning. It is not clear, however, whether this shift in response to experience is in fact a learned response or a biological clock adjustment based on physiology.

In short, the mechanisms underlying effects of prior experience on mating behavior can be difficult to distinguish, particularly in systems where experience mediates physiological change. Distinguishing between alternative mechanisms would give us a greater understanding of how different social, physiological, and environmental

variables impact specific components of mating behavior, yet few studies have done this in a systematic fashion.

In a study of a walnut-infesting tephritid fly (*Rhagoletis juglandis*), we examined the effects of host fruit experience and physiological state on mating behavior. We manipulated physiological state in terms of egg load (defined as the number of mature oocytes in a female's ovaries) independently of fruit experience to distinguish an effect of experience on mating behavior due to egg load from other effects of experience. This system was of interest in part because exposure to host fruit is known to stimulate oogenesis and enhance egg load in females (Alonso-Pimentel et al., 1998) and because egg load was known to influence other aspects of a female's reproductive behavior (Alonso-Pimentel and Papaj, 1996a). We argue that distinguishing between these variables may be a way to gain insight into the mechanisms underlying mating decisions, as well as providing insight into functional aspects, such as sexual conflict over different components of mating behavior.

Methods

Study system

Like other members of this genus, *R. juglandis* is characterized by a resource-defense mating system, in which the males engage in territorial contests over walnut fruit. Females oviposit and larvae develop solely in the fruit. Females arriving at the fruit often mate during oviposition attempts (Papaj 1994) in what sometimes appear to be "forced" copulations. Females mate with multiple males, and last male sperm precedence has been

shown for this genus (Opp et al., 1990, 1996). Previous work with *R. juglandis* (Alonso-Pimentel and Papaj, 1996b) has shown that copulation duration is variable in this species, with longer matings occurring when the operational sex ratio is male biased, and shorter copulations occurring when the population is female biased. Longer copulations are thought to benefit the male as a form of mate guarding. In southern Arizona, these flies exclusively use Arizona walnut, *Juglans major*, as a host plant, emerging between July and September from puparia in the soil. For this study, we used flies collected as larvae at six sites in central and southern Arizona in 2002. Pupae were maintained at 4 ^{o}C prior to the study, and warmed to room temperature (ca. 29 ^{o}C) for approximately 5 weeks before eclosion.

Experiment 1

We separated flies by sex within four days of eclosion and placed them into clear plastic 473-ml cups containing water, yeast extract, and sugar cubes. Previous studies have indicated that sexual maturity of both sexes of *Rhagoletis*, and the onset of copulation, is not achieved until at least 6-8 days post eclosion; therefore, we are confident that flies used in our study were virgins (Boyce 1934; Prokopy et al. 1972; Webster and Stoffolano 1978; Alonso-Pimentel and Papaj 1996a). Flies were held in cups for seven to eight days prior to testing. We created groups of high and low egg load females by exposing one group of females to a surrogate fruit (a 3.7-cm diameter yellow plastic sphere) during the holding period. Males and control females were not exposed to fruit until the day of testing. This procedure causes experimental females to mature more oocytes faster than

control females (Alonso-Pimentel et al., 1998). All holding cups were surrounded by white cardboard barriers to control for effects of extraneous visual stimuli.

On the day of testing, we isolated 10 males individually in clear plastic observation cups with a surrogate fruit suspended from the top of the cup. We randomly paired half of the males with a fruit-exposed female and half of the males with a control female for one hour, and observed: 1) whether or not copulation occurred, and 2) the duration of the first copulation in each cup, if a copulation occurred. At the end of each test, all flies were frozen. We measured body size in all females because egg load in many insects, including this species, is positively correlated with body size (Alonso-Pimentel, 1998). We additionally measured body size in males, reasoning that mating traits could conceivably vary with male size. Wing vein measurement was recorded for both males and females as a proxy for size, as this measurement correlates well with body mass (mid-wing vein length; Alonso-Pimentel, unpublished data). We subsequently dissected females and counted the number of mature oocytes contained in the ovaries. Data were analyzed using JMP-IN statistical software (SAS Inc.; Cary, North Carolina).

Experiment 2

We conducted a second experiment in order to manipulate egg load independently of fruit experience. In this experiment, flies were again separated by sex as above, within 4 days post-eclosion. We created groups of high and low egg load females by treating experimental flies with the juvenile hormone analogue methoprene. In flies, rising juvenile hormone titers trigger oogenesis, and topically-applied methoprene has been

shown to have virtually identical effects (Duan et al., 1995). We topically applied 1uL of 50uM methoprene (technical grade, greater than 95% purity, Zoecon Corp.; dissolved in acetone) to the abdomen of each fly in the experimental group using a microsyringe (10 uL syringe, Hamilton Co, Reno, NV). Control flies were treated with 1uL of acetone in the same manner. Prior to methoprene or acetone-only application, all flies were exposed to gaseous carbon dioxide for 30 seconds in order to facilitate handling. Again, flies were held for 7-8 days prior to testing, with conditions as in Exp. 1 except that no surrogate fruit was present in any of the holding cups. Mating trials were conducted as above, with a surrogate fruit present.

Results

Experiment 1—Effects of surrogate fruit exposure

In experiment 1, we examined the effects of fruit exposure by exposing experimental flies to surrogate fruit. We conducted trials on a total of 190 pairs of flies. Mean body size of both females and males, as measured in terms of wing mid-vein length, was not significantly different between treatments (ANOVA: for females, mean = 1.62mm, SE = 0.01mm for both treatments, $F= 0.193$, $p= 0.66$; for males, mean = 1.52mm, SE = 0.01mm for both treatments, $F= 0.125$, $p= 0.72$). Mean egg load was significantly higher for females exposed to fruit than for control females (ANOVA: control mean = 15.7, SE = 2.0, fruit-exposed mean= 35.9, SE = 2.0, $F= 49.2$, $p<0.0001$). In mating trials, copulations were more frequent when females had higher egg loads (Fig. 1). The results of a nominal logistic regression revealed that, although fruit exposure influenced egg

load, likelihood to copulate depended on egg load directly and was independent of fruit exposure treatment (Table 1).

Copulation duration was strongly bimodal, with all copulations in both experimental and control groups lasting 400 seconds or less, or 600 seconds or more (Fig. 2). We therefore analyzed copulation duration as a nominal variable, where duration was either short or long. Copulation duration also differed between treatments, with pairs containing experimental females copulating for significantly shorter durations than control females (For control, 42% of copulations were short, for fruit-exposed, 70% were short; $\chi^2 = 5.1$, $p = 0.02$). However, in contrast to the results for likelihood to copulate, a nominal logistic regression revealed that fruit exposure itself, rather than egg load per se, accounted for this difference in duration (Table 2).

Experiment 2 – Effects of methoprene application

In experiment 2, we examined effects of egg load on mating behavior independently of fruit exposure by exposing experimental flies to methoprene, a juvenile hormone analogue known to induce oogenesis. We conducted trials on a total of 150 pairs of flies. As in the first experiment, mean body size of both females and males, as measured by the length of the wing mid-vein, was not significantly different between treatments (ANOVA: for females, mean =1.61mm, SE = 0.01mm for control, mean length =1.62mm, SE = 0.01mm for methoprene, F=0.33, p=0.56; for males, mean=1.52mm, SE = 0.01mm for both treatments, F=0.003, p=0.95). Mean egg load was significantly higher for methoprene-treated than untreated females (ANOVA: control mean = 14.4, SE = 2.2,

methoprene mean= 39.3, SE = 2.2, F= 65.2, $p<0.0001$). As in experiment 1, copulations were more frequent for females with a higher egg load (Fig. 1), and again, this effect was independent of treatment (Table 3).

Copulation duration was again strongly bimodal in this experiment (Fig. 2). However, in contrast to experiment 1, copulation duration did not depend on treatment; copulation duration was not significantly different between pairs with methoprene-treated vs. untreated females (For control, 48% of copulations were short, for methoprene, 44% were short; $\chi^2 = 0.075$, $p = 0.78$). Egg load also had no effect on copulation duration.

Discussion

In behavioral ecology studies of foraging, it has been known for some time that effects of experience and physiological state can be confounded. Rosenheim and Rosen (1991), for instance, teased apart the effects of egg load and prior host experience on host-acceptance behavior in a parasitoid wasp (see also Henneman et al., 1995). Given a growing number of studies indicating that a female's prior experience as well as her physiological state influence mating behavior (see Introduction above), it is important to address this issue in the context of mating dynamics.

The present study found evidence that both physiological state, in terms of egg load, and host experience independent of egg load, affect mating behavior. Moreover, different components of mating behavior responded to resource experience and physiological state in different ways. Whether or not a pair copulates was dependent on egg load, with greater propensity to copulate occurring among females with higher egg

loads, whether high egg load was induced via methoprene or fruit experience. A female's experience with host fruit did not affect propensity to copulate except as it enhanced egg load (i.e., an indirect route of experiential effects indicated by the bold arrows in Fig. 3A). In contrast, the length of a copulation depended on a female's experience with host fruit. This effect was not mediated via effects on egg load, as egg load per se had no effect on copulation duration. Evidently, some other mechanism related to host experience, possibly learning, was at play (i.e., a potentially direct route of experiential effects indicated by the bold arrow in Fig. 3B).

The results of experiment 2 support the inferences from experiment 1 that a female's experience with fruit, rather than egg load per se, accounts for differences in copulation duration, whereas egg load, not host experience per se, accounts for differences in propensity to mate. The consistency of results across experiments is critical to the strength of our inferences. In particular, inferences based on statistical patterns in experiment 1 potentially lack robustness, as they are contingent upon the validity of assumptions about sources of error. For example, the fact that egg load explains a significant amount of variation in likelihood of copulation, whereas fruit exposure independent of egg load does not, may reflect a biological difference, as we infer and as is supported by experiment 2. However, the same pattern might also be obtained if, for instance, egg load was measured with less error than fruit exposure treatment was "measured." We feel that this difference in measurement error is unlikely; if anything, errors in measuring egg load are more likely, not less likely, than errors in measuring

treatment, particularly as we isolated the effects of fruit exposure by using blinds around all cups in both experiments.

The fact that physiological state and prior experience can influence different aspects of behavior in different ways underscores the importance of distinguishing these variables. In addition to providing potential insights about mechanism, the type of variable impacting a specific component of behavior holds implications for the functional significance of that behavior; in particular, examining each variable separately may reveal patterns of sexual conflict over mating. While we can only offer speculation on the functional significance of the patterns in this study at this point, even speculation illustrates the importance of understanding the route by which experience exerts effects on behavior.

Functional aspects: Physiological state

With respect to physiological state, in this case egg load, both female and male perspectives would seem to dictate that females of relatively higher egg load will copulate relatively more readily, as we found. This effect could reflect increased mating effort by the male, the female, or both sexes. Because oviposition facilitates oogenesis (reviewed in Papaj 2000), females with high egg loads and available sperm can rapidly lay eggs and go on to develop multiple clutches, resulting in high fecundity. Females that maintain high egg load without possibility to lay fertile eggs, therefore, may pay a fitness cost in terms of reduced lifetime fecundity. From a virgin female perspective, this scenario would favor an increased propensity to copulate, perhaps even at the expense of

mating with a low-quality male. Although we used virgins in our study, these results could also apply to already-mated females, as they could also potentially benefit from copulating more readily when many eggs are mature (direct benefits, genetic diversity, and genetic compatibility, to name three; see Vahed, 1998; Jennions and Petrie, 2000).

The increase in propensity to copulate when females carry a high egg load is consistent with the male perspective, as well. Studies have shown that males prefer to mate with females in better overall condition (Danielson-Francois et al., 2002) or advanced reproductive state (Kelso and Verrell, 2002). In particular, several studies with insects have shown that males prefer females with relatively larger abdomens, presumably a cue that she carries a higher egg load (reviewed in Bonduriansky 2001). In walnut flies, males may also improve their chances of siring offspring by mating with high egg load females, as such females are likely to lay large clutches relatively quickly, and prior to re-mating. This advantage is particularly meaningful given the form and intensity of sperm competition in this species: mating in the genus *Rhagoletis* and in other tephritid flies has been uniformly shown to be last male precedence (Opp et al.,1990; Yamagishi et al., 1992; Saul and McCombs, 1993; Opp et al., 1996), and female *R. juglandis* re-mate frequently in nature (D. Papaj, pers. obs.).

While the perspectives of both sexes appear to be congruent in terms of propensity to copulate with respect to egg load, the perspectives of the two sexes over copulation duration with respect to egg load appear to diverge. From a male perspective, a female with a high egg load may be a more valuable reproductive resource. Therefore, a male might engage in long copulations with high egg load females for at least three

reasons: 1) to preclude other males from mating with the high quality female through contact mate guarding, 2) to ensure sufficient sperm transfer to fertilize all eggs, which may prevent a female from remating due to sperm depletion, or 3) to transfer sufficient sperm to flood out previously deposited sperm from rivals (Simmons, 2001). In contrast, from a female perspective, a female with a high egg load should copulate for a shorter duration, as long copulations could be costly in terms of reducing time available for laying eggs (Sherman, 1983). When females mate with multiple males, optimal mating strategies, including copulation duration, may differ between the sexes, and thus, sexual conflict over reproductive decisions may arise (Stutt and Siva-Jothy, 2001). The observed absence of an association between copulation length and egg load in our study may be the net result of such conflict (cf. Simmons 2001).

Functional aspects: Prior experience

With respect to effects of prior experience independent of egg load (for instance, effects reflective of learning), a female perspective would seem to favor an increased propensity to copulate and shorter copulation duration for fruit-exposed females. One effect of fruit experience could be to provide information to the female about the quality and availability of host fruit. A fruit-exposed female has learned that high quality fruit are consistently available, and thus, she would benefit from obtaining sperm quickly and allocating more time to laying eggs.

In contrast, males in experiment 1 were uniformly held without fruit prior to testing. Because male experience was not manipulated, the male perspective for

propensity to copulate and for copulation duration should be neutral with respect to female experience. As such, our results with respect to prior experience reflect a congruence with the male perspective in terms of propensity to copulate (fruit experience did not determine propensity, consistent with the neutral perspective), and with a female perspective in terms of copulation duration (copulations were shorter for fruit-exposed females). This pattern implies that, in relation to effects of experience, copulation duration was under female control, but the decision to copulate in the first place was under male control. Evaluating these inferences will require some independent means for assessing gender control over mating traits.

General implications

As illustrated in the sections above, the perspective of an individual from the standpoint of changes in physiological state is not necessarily congruent with its decision from the standpoint of an experiential process such as learning. Thus, understanding whether behavioral plasticity stems from changes in physiological state per se or an experience-related change such as learning can be important in understanding the function of behavioral traits. While teasing apart these potentially confounding effects could hold implications for many kinds of behavior, mating provides a particularly interesting focus, as the fitness consequences for mating decisions can differ between genders. We argue that this approach may be an effective way to gain insight into which sex is in control of a specific aspect of mating behavior.

In closing, we note that a long tradition in experimental psychology has wrestled with the issue of interactions between experience and physiological state, the latter commonly referred to as “motivation.” A clearer understanding by behavioral ecologists of the fitness consequences of behavioral change with experience is sure to benefit from the approaches developed in that discipline.

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Figure Legends

Figure 1. Propensity to copulate was higher when females had higher egg loads ($p < 0.01$ for exp. 1; $p < 0.0001$ for exp. 2; see Tables 1 & 3 for full results of model). Results from exp. 1, fruit exposure, are shown in the top panel, while results from exp. 2, methoprene exposure, are shown in the bottom panel.

Figure 2. Copulation duration was strongly bimodal in both exp. 1 and exp. 2; thus, duration was analyzed as either short (400 s. or less) or long (600 s. or more). Results from exp. 1, fruit exposure, are shown in the top panel, while results from exp. 2, methoprene exposure, are shown in the bottom panel.

Figure 3. Egg load and prior experience have different effects on different components of mating behavior. Part A: egg load directly determined propensity to copulate, whether high egg load was induced via fruit exposure or methoprene. Prior experience had no direct effect on propensity to copulate, but had an indirect effect by increasing egg load in exp. 1. Part B: Prior experience had a direct effect on copulation duration, but egg load had no effect.

Table 1. Results of nominal logistic regression model for propensity to copulate in experiment 1, fruit exposure.

	Estimate	Std. Error	Chi Square	P-value
Intercept	1.180	0.305	14.93	0.0001
Fruit exposure	0.238	0.190	1.57	0.21
Egg load	-0.027	0.009	8.32	0.0039
Fruit exposure * Egg load	0.013	0.009	2.19	0.14

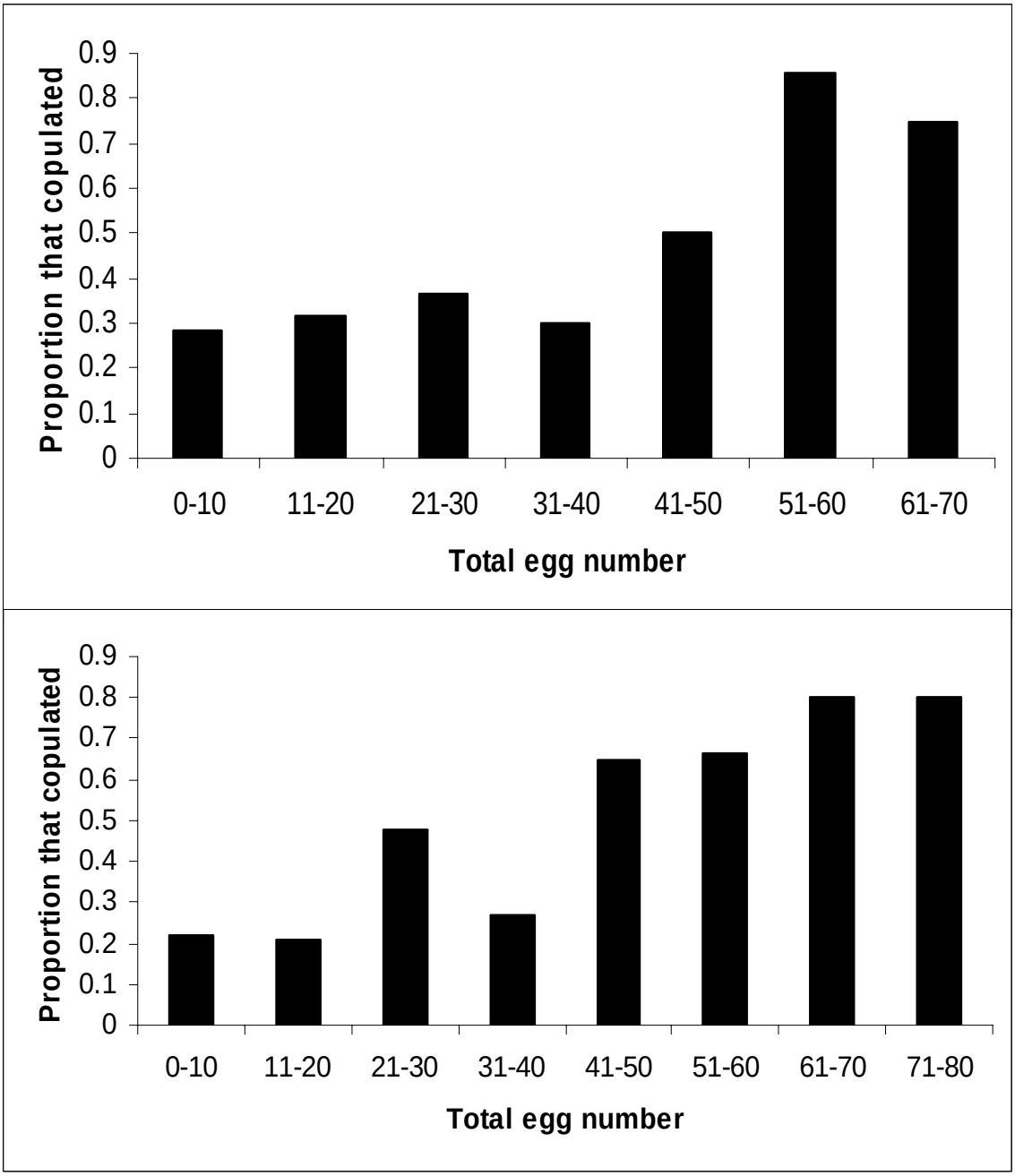
Table 2. Results of nominal logistic regression model for copulation duration in experiment 1, fruit exposure.

	Estimate	Std. Error	Chi Square	P-value
Intercept	0.47	0.519	0.83	0.36
Fruit exposure	-0.708	0.333	4.53	0.03
Egg load	-0.007	0.015	0.28	0.59
Fruit exposure* Egg load	0.002	0.015	0.01	0.90

Table 3. Results of nominal logistic regression model for propensity to copulate in experiment 2, methoprene.

	Estimate	Std. Error	Chi Square	P-value
Intercept	1.745	0.386	20.39	<0.0001
Meth exposure	-0.037	0.234	2.48	0.11
Egg load	-0.051	0.012	19.18	<0.0001
Meth exposure * Egg load	-0.007	0.011	0.35	0.55

Figure 1.


Proportion that copulated
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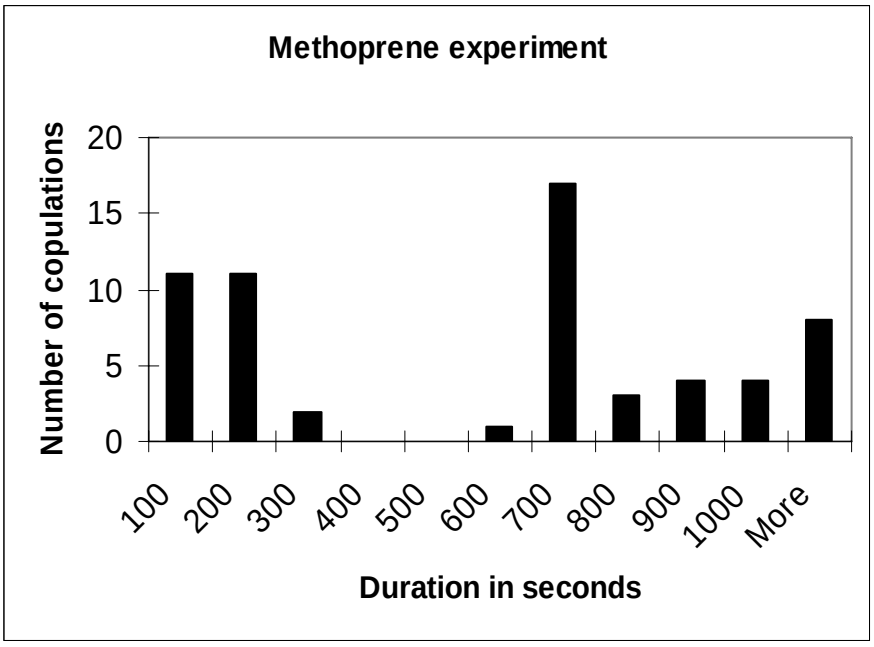
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0-10
11-20
21-30
31-40
41-50
51-60
61-70
Total egg number
Proportion that copulated
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
Total egg number

Figure 2.


Methoprene experiment
Number of copulations
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10
15
20
100
200
300
400
500
600
700
800
900
1000
More
Duration in seconds

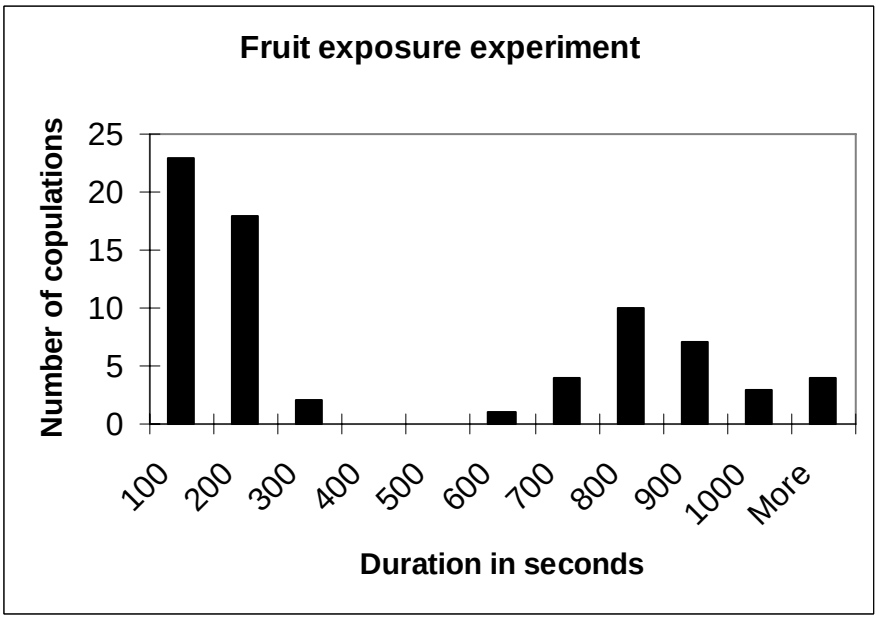
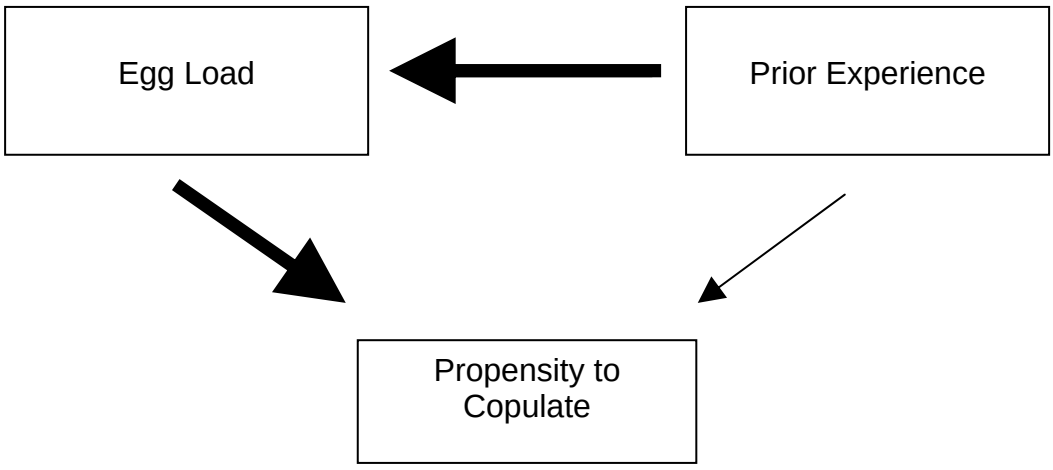

Fruit exposure experiment
Number of copulations
0
5
10
15
20
25
100
200
300
400
500
600
700
800
900
1000
More
Duration in seconds

Figure 3.

Part A.



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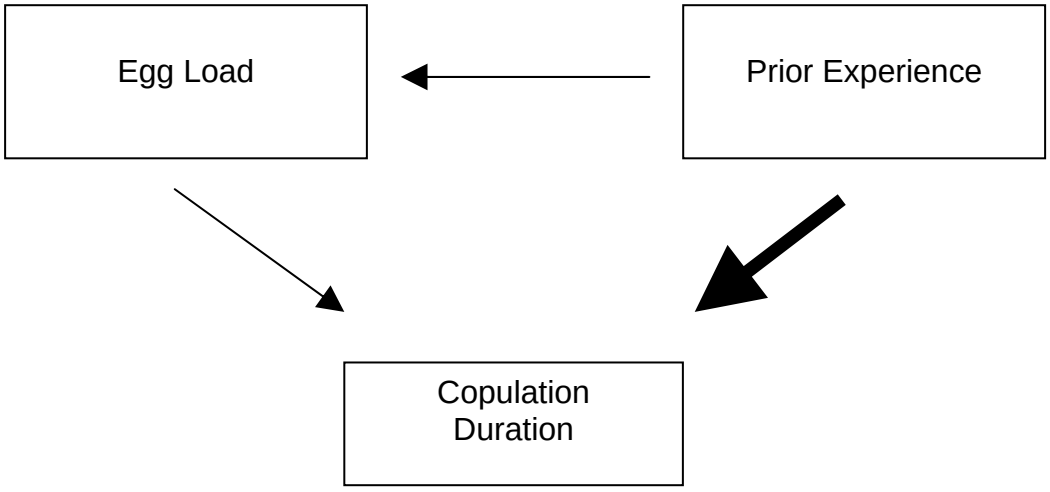

APPENDIX B

SEX RATIO DURING DEVELOPMENT DETERMINES TESTES SIZE IN A TEPHRITID FLY

Title: Sex ratio during development determines testes size in a tephritid fly

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Abstract

Traits such as testes size are thought to experience strong selection due to sperm competition. At the interspecific level, species that experience strong sperm competition generally have evolved larger testes than those experiencing weak sperm competition. However, levels of sperm competition may vary in time in space, leading to selection for flexibility in testes size within a species. Such adjustment of testes size by individuals has rarely been investigated. In this study, we asked whether males could adjust testes size in response to local levels of sperm competition, as indicated by social environment. We manipulated the sex ratio of the tephritid fly, *Rhagoletis juglandis*, during adult development and measured fully developed testes size. As expected, testes size was in part a function of body size. However, males reared in more male-biased sex ratios developed larger testes than males reared in more female-biased sex ratios, independent of body size. Behavioral observations of flies indicated that per capita rates of contest behavior increase when the sex ratio is male biased, and that per capita mating effort decreases. An additional experiment showed that testes size adjustment occurs in response to sex ratio independently of mating activity. Taken together, our results suggest that male flies can respond dynamically to their social environment by allocating more resources to testes when sperm competition is expected to be more intense. This type of flexibility may allow a male to appropriately tailor investment into various tissues and activities depending on the level of sperm competition that he will experience. While behavioral flexibility in response to variability in sex ratio is a relatively common strategy, this is the first report of flexibility in allocation to a reproductive organ in

response to sex ratio. We discuss conditions that might favor the evolution of this strategy.

Key words: sperm competition; testes; sex ratio; social environment; tephritid; walnut fly

Introduction

When females mate with multiple males during a single reproductive episode, sperm from different males compete for fertilization of eggs within the female reproductive tract. Because female remating is widespread in nature (rev. in Birkhead & Møller 1998; Simmons 2001), the outcome of sperm competition has important fitness consequences for males in many species.

Theory predicts that males should invest more heavily into ejaculates under scenarios where the levels of sperm competition are high (Parker 1998). Increased investment may take the form of larger ejaculates, ejaculates of better quality, or increased rate of sperm production (Hosken *et al*. 2001; Preston *et al*. 2003; Schulte-Hostedde & Millar 2004). Because testes size often predicts ejaculate size and quality (e.g. Møller 1989; Stockley & Purvis 1993; Pitnick & Markow 1994), as well as sperm production rate (Schärer *et al*. 2004), it follows that testes size should increase in response to increased sperm competition.

A number of empirical studies support the predicted pattern. Polyandrous species which mate multiply or live in large colonies tend to have larger testes than monogamous species or species living in smaller colonies (Svärd & Wiklund 1989; Møller 1991; Gage 1994; Stockley *et al*. 1997; Pitcher *et al*. 2005). Selection studies report a similar pattern. In yellow dung flies, males from lines in which polyandry was permitted had larger testes than males from lines in which monogamy was enforced (Hosken *et al*. 2001).

In the studies cited above, differences in testes size among species or lines are considered to be evolved. Alternatively, it is conceivable that testes size is adjusted

ontogenetically in response to local levels of sperm competition. Ontogenetic adjustments are expected when the level of sperm competition varies over time and space in a way that cannot be predicted in advance by a developing male. For instance, cockroaches (*Nauphoeta cinera*) produce larger spermatophores in the presence of other individuals then when housed alone (Harris and Moore 2004). However, there is limited evidence that individuals can adjust testes size ontogenetically in response to level of sperm competition. In the present study, we examined whether testes development and, in particular, the size of testes at developmental maturity, was influenced by level of sperm competition. We focused on the role of an ecological factor, operational sex ratio, of known importance in mate competition, but never studied previously in the context of testes size adjustment. Operational sex ratio (OSR) is defined as the ratio of the frequency of sexually active males in a population to the frequency of receptive females (Emlen & Oring 1977). More male-biased operational sex ratios are known to cause males to behave as if levels of sperm competition were higher. For example, both male-male contest behavior (e.g. Enders 1993; Kvarnemo *et al*. 1995) and mate guarding activity (e.g. Jormalainen *et al*. 1994; Alonso-Pimentel & Papaj 1996*a*, 1999; Schofl & Taborsky 2002) intensify under a male-biased sex ratio. Ejaculate size and sperm expenditure (e.g. depletion of sperm reserves) may increase under these conditions as well (e.g. Evans *et al*. 2003; Garcia-Gonzalez & Gomendio 2004). No study to our knowledge has demonstrated variable investment in a male reproductive organ in response to a biased OSR.

The walnut fly, *Rhagoletis juglandis*, is an ideal model system for examining the effect of OSR on testes size. There is a high degree of multiple mating among both males and females, and thus ample opportunity for sperm competition. In addition, testes are large organs, filling about a third of the abdomen (L. Carsten-Conner, pers. obs.) and thus are likely to be energetically expensive (see Dewsbury 1982; Wedell *et al*. 2002).
It is more likely that allocation to expensive structures or functions will vary, as life history theory predicts that variation in allocation will be driven in part by the cost of investment (Roff 1992; Stearns 1992). Finally, individuals of this species are known to adjust their behavior in response to changes in OSR. Previous work (Alonso-Pimentel & Papaj 1996*a*, 1999) has shown that copulation duration varies in relation to OSR, with longer copulations occurring more frequently when OSR is male-biased. On the basis of testes investment considerations alone, one might expect that male walnut flies would develop larger testes at maturity as OSR became progressively more male-biased.

One might have an altogether different expectation if one takes into account investment into other male traits. For example, it is possible that investment in testes trades off against investment in contest behavior (Simmons & Emlen 2006). Male-male contests are a central feature of the walnut fly mating system, determining a male's capacity to monopolize a fruit and thereby gain access to females coming to the fruit to oviposit (Papaj 1994). Although these contests do not result in any obvious physical damage to the flies, they may constitute "wars of attrition" that are energetically expensive (Marden and Waage 1990, Hack 1997, DeCarvalho et al. 2004). If investment in testes and contests are both expensive, then allocation to one function might limit

allocation to the other under an increasingly male-biased sex ratio. It is conceivable that an increase in investment in contests under increasingly male-biased sex ratios might offset increased investment in testes. Therefore, we also examined how contest behavior changed with changes in sex ratio.

Methods

Experiment 1

Flies were collected as larvae in Santa Cruz County in southern Arizona in August of 2004. Pupae were maintained at 4^{o}C prior to the study, and warmed in a growth chamber (ca. 28^{o}C) until eclosion approximately 4 weeks later. Once flies eclosed, they were reared in a growth chamber kept at ca. 28^{o}C. Within 48 hours of eclosion, we aspirated flies into rearing cups (clear plastic 473-ml cups containing water, yeast extract, and sugar cubes) at one of five different sex ratios: 1M:11F, 3M:9F, 6M:6F, 9M:3F, or 12M:0F. These regimes held overall fly density constant while varying sex ratio. Flies were prevented from visual access to other cups by means of white cardboard barriers. Each of the latter four ratios were replicated three times, and the 1M:11F ratio was replicated six times, for a total of 18 rearing cups (the 1M:11F ratio had a higher number of replications in order to increase the sample size of males at that sex ratio). Flies were held in these cups for 16 days in order to ensure that testes had reached maximal growth at the time of dissection (*R. juglandis* testes increase in size for approximately 11 days after eclosion; L. Carsten-Conner, unpublished data), then killed by freezing. We repeated the experiment three weeks later exactly as described above, except that flies

were frozen after 13 rather than 16 days (this was done for logistical reasons). Both replicates of the experiment were conducted in the same growth chamber, under a consistent light regime (12 hours light:12 hours dark), and under low humidity conditions (ca. 20%).

Male flies were dissected in glass depression slides with a drop of Ringer's solution (0.9g NaCl, 0.02g KCl, 0.4g dextrose, and 0.02g $CaCl_2$ in 100 ml deionized water) within 24 hours of freezing. We used the area of digitized images of testes as our size metric. Area is a commonly used measurement of testes size in insect studies, and has been shown to accurately represent the three-dimensional size of testes (Minder et al. 2005). Both testes of each male were transferred to a compound glass slide and analog video was recorded at 50x magnification under a dissecting scope. Analog images of the testes were captured and digitized, and ImageJ software (National Institutes of Health, version 1.32j) was used to estimate the total area encompassed by both testes. We used the mean area of the two testes in a given male as the size metric. Mid-wing vein length was also recorded for each animal as a proxy for body size, as this measurement correlates well with body mass (H. Alonso-Pimentel, unpublished data). In order to equalize sample sizes, we dissected all of the males in the 1M:11F, 3M:9F, and 6M:6F cups, but in the 9M:3F and 12M:0F cups, a subset of only 6 males was chosen randomly for dissection.

We used a linear mixed-effects model fit by REML to ask whether mean testes area was predicted by sex ratio and/or body size. Sex ratio and body size were included in the model as fixed effects, while rearing cup was included as a random effect. This model

was more appropriate than a nested analysis (nesting cup within sex ratio), because in the 1M:11F ratio there was only one male per cup, making nesting impossible. Testes area was log transformed prior to analysis to normalize the residuals. We also used a linear model to generate residual testes mass after removing body size; the residuals were regressed against sex ratio. Data were analyzed using JMP-IN statistical software (SAS Inc.; Cary, North Carolina, version 5.1.2.

In order to account for possible effects of rearing cup on our results, we performed another full analysis with a linear model, asking if testes area was predicted by sex ratio, body size, or cup nested within sex ratio. We used a separate analysis to account for these effects because a nested analysis could not be performed on the entire data set; in the 1M:11F ratio there was just one male per cup. Thus, we excluded observations of testes size at this ratio from the nested analysis.

Experiment 2

We concurrently carried out a second experiment that examined frequency of contest behavior between males. Pupae were maintained as above. Within 48 hours of eclosion, we aspirated flies into rearing cups at one of four different sex ratios: 3M:9F, 6M:6F, 9M:3F, or 12M:0F. Each sex ratio was replicated 3 times for a total of 12 cups. Flies were prevented from visual access to other cups by means of white paper barriers. We observed the cups for 7 consecutive days, beginning with the second day after eclosion. Flies were observed every hour during hours of peak activity, from 10 AM until 2 PM. We recorded behavioral interactions in each cup for two minutes, then moved on

to the next cup. All intrasexual interactions, including male-male wing displays, male-male mounting, chasing, and actual combat (males rear up and spar with front legs) were recorded. We pooled these data for an overall index of contest behavior. We also recorded all intersexual behavior in a cup. Courtship display, mounting attempts, and successful mating attempts were recorded and pooled to obtain an overall index of mating-related behavior. We calculated per capita frequency of contest behavior and per capita frequency of mating behavior for each cup in each treatment and conducted ANOVA to determine whether sex ratio affected the frequencies of contest and mating behavior. We included cup nested within sex ratio as a factor in the analysis. All behavioral data are presented as proportions, log transformed to normalize the residuals. Because of the high number of zeroes in the data set, the transformation took the form of log [prop. behavior + 1].

Experiment 3

Because observations in Experiment 2 indicated that males engaged in fewer per capita matings in male-biased sex ratio cups than in female-biased sex ratio cups (see results), we conducted a third experiment to address the possibility that differences in testes size in relation to sex ratio were mainly a consequence of differences in mating frequency. The primary objective of this experiment was to manipulate the possibility of mating at a given sex ratio and determine if testes size varied with mating. We included controls to determine if the effect of sex ratio on testes size persisted even when mating was physically prevented.

The experiment was conducted at the University of Alaska, in a rearing chamber with similar temperature, humidity, and light conditions as those at the University of Arizona (ca. 28^{o}C, 20%, and 12 hours light: 12 hours dark, respectively).

To determine how mating per se affected testes size at a given sex ratio, we aspirated flies into 18 plastic rearing cups as described above, at a 2M:10F (female-biased) sex ratio. We chose a female-biased ratio because males would be expected to experience a high frequency of mating at this ratio, giving us more power to detect an effect of mating on testes size. We chose a 2M:10F ratio rather than the 1M:11F ratio used in Experiment 1 in order to increase the sample size of males for analysis (space constraints in the rearing chamber precluded simply adding more cups to the experiment). Mating was prevented in half of the cups, by means of a clear plastic barrier that divided the cups into two portions. Males were contained in one half of the cup and females in the other half, each with access to sugar, protein, and water as described above. Because the plastic barrier was clear, males could obtain visual cues about the number of females in the environment. We also ventilated the plastic barrier to allow dispersion of any possible pheromones between the two sides of the cup. Flies were prevented from visual contact with other cups by means of white paper barriers. Flies were held in these cups for 13 days, then frozen and dissected. Wing vein length was recorded and testes were photographed at 50x under a dissecting microscope.

To get a preliminary assessment as to whether the sex ratio effect on testes size persisted even in the absence of mating, we also included four 9M:3F (male-biased) cups in the experiment. In these cups, mating was prevented by a physical plastic barrier, as

described above. We chose the 9M:3F ratio because results of Experiment 1 indicated that the contrast between testes size was strongest between the 9M:3F and the 1M:11F sex ratio treatments (see results).

Because this data set contained more than one male in every cup, a nested analysis was appropriate. We used a linear model that asked if testes area was predicted by group (male-biased sex ratio with no mating, female-biased sex ratio with no mating, or female-biased sex ratio with mating allowed), body size, or cup nested within group. The residuals were normally distributed, obviating the need for log transformation. Data were again analyzed using JMP-IN statistical software (SAS Inc.; Cary, North Carolina, version 5.1.2).

Results

Experiment 1

Mean body size, as measured by mid-wing vein length, in Experiment 1 was 1.36 ± 0.01 mm. Overall mean testes size was 0.28 ± 0.01 mm^2. Body size strongly predicted testes size, with larger males generally having larger testes (Figure 1). Sex ratio also strongly predicted testes size (Figure 2), largely due to the effects of large testes size in the 9M:3F cups. After removing body size and plotting the residuals against sex ratio, the effect of sex ratio is stronger, with testes size generally increasing as sex ratio becomes more male-biased (Figure 3). The sex ratio effect is not a consequence of body size: first, the model assessed the effect of sex ratio independent of the effect of body size and, second, in a separate analysis mean body size was not significantly different across sex ratio

treatments ($F_{4,118} = 0.67$, $p = 0.61$). Because cup was treated as a random effect, there was no associated p-value with the REML model. The nested analysis showed that cup was significant; however, after accounting for cup effects, body size and sex ratio both remained highly significant.

Experiment 2

The per capita frequency of contest behavior per cup depended on sex ratio (Figure 4). The overall effect of sex ratio on frequency of contest behavior is due to the difference between the 3M:9F treatment and each of the other more male-biased treatments. Cup was not a significant effect. Per capita mating effort also varied with sex ratio (Figure 5), but in contrast to contest behavior, the per capita frequency of mating behavior per cup declined as sex ratios became more male-biased. Cup was not a significant effect.

Experiment 3

Mean testes area was again correlated with mid-wing vein length, an estimator of body size, with larger males tending to have larger testes (Figure 6; Mean body size, as estimated by mid-wing vein length, was 1.45 ± 0.01 mm in Experiment 3, while overall mean testes area was 0.43 ± 0.01 mm^2). Treatment (2M:10F no mating, 2M:10F mating, or 9M:3F no mating) also strongly predicted testes size (Figure 7). The difference in testes size was due solely to sex ratio: there were no differences in mean testes size between the female-biased sex ratio with mating allowed and the female-biased sex ratio with mating prevented, but the male-biased sex ratio group differed from both (Figure 7).

There was no effect of rearing cup. To further test our inferences about the relationship between testes size, OSR, and mating, we conducted a least-square means contrast between the 2M:10F mating allowed and the 2M:10F mating prevented treatments. As expected, there was no significant difference between these treatments ($t = 0.1165$, $p = .91$), further suggesting that testes size does not vary with opportunity to mate for a given sex ratio. We conducted the same test between the 9M:3F treatment (in which mating was prevented) and the 2M:10F. In contrast, this effect was highly significant ($t = -3.302$, $p = 0.002$), supporting the inference that the OSR effect on testes does not require mating.

Discussion

To our knowledge, these results are the first to show that testes size is influenced by the operational sex ratio that males experienced as their testes matured. There are few studies that demonstrate ontogenetic adjustment of testes size in response to local levels of sperm competition. Some studies have shown that testes size correlates positively with social group size (Brown & Brown 2002 in cliff swallows; Gage 1995 in a moth; and Tan *et al*. 2004 in leeches). However, sex ratio is expected to be a more precise predictor of level of sperm competition than is overall density (Emlen & Oring 1977). Given that testes size is positively correlated with sperm competitive ability (e.g. Møller 1989; Stockley & Purvis 1993; Pitnick & Markow 1994; Schärer *et al*. 2004), our results suggest that male walnut flies that adjust testes size in the manner described here will be at an advantage during episodes of multiple mating by females, particularly under male-biased sex ratios.

The ontogeny of this allocation-based response to social setting differs markedly from more commonly described behaviorally-based responses, such as mate guarding, extended copulations, or transfers of large ejaculates. The latter responses tend to occur rapidly and are generally reversible. For example, in assays of walnut flies, an observed effect of OSR on copulation duration (Alonso-Pimentel & Papaj 1996*a*, 1999) required only hours or even minutes to be expressed (Alonso-Pimentel & Papaj 1996*a*), and could be reversed within a day or less by placing individuals in a new OSR regime.

In contrast, allocation to testes is a relatively slow process. Newly eclosed males require approximately five days on average to reach sexual maturity, and testes do not reach their full size until about 11 days post eclosion, regardless of the sex ratio regime in which they are held. These patterns suggest that any significant change in testes size in response to sex ratio occurs on the order of days, rather than hours or minutes. Although we do not have information on the reversibility of the OSR effect on testes, any reversal that does occur is also likely subject to this longer time scale.

Speedy and reversible responses are advantageous because they allow males to more easily track changes in typical reproductive environments, which are dynamic on the order of minutes or hours. A much slower response is functional only when environmental change takes place on a longer time scale. For walnut flies, OSR varies over at least two time scales. In the vicinity of an individual fruit in a tree, OSR might change within minutes or hours. The observed adjustment in copulation duration appears to operate effectively over that time scale (Alonso-Pimentel & Papaj 1996*a*), but the adjustment in testes size would not. However, OSR within the host tree as a whole varies

over a longer time scale, on the order of weeks (Alonso-Pimentel & Papaj 1996*b*). In field surveys of walnut trees, OSR was consistently male-biased within the host tree throughout the one-month flight season. However, the degree of male bias increased sharply during the first part of the season, and then decreased progressively in the latter part of the season. Because males emerge more or less continuously for at least the first two weeks, there may be significant variation in the OSR environment in which males are maturing their testes. This variation, if unpredictable from the standpoint of an individual male, may favor a capacity to adjust testes size, such that males eclosing when there is a more male-biased sex ratio, and thus more sperm competition, might develop larger testes than males eclosing at other times.

Testes size adjustment should be favored only if testes maturation can reliably track variation in OSR. That is, males need to be able to 'predict' what the OSR will be at the time of testes maturity and allocate investment in testes accordingly. Since sexual maturation takes 5 or more days, the critical issue is whether or not OSR at maturation can be predicted accurately at the time that a testes size allocation 'decision' is made. An answer to this question will require more detailed knowledge of timing and rate of testes development. We also need to know more about variation in OSR among host trees and sites in order to determine if testes size can effectively track OSR in nature.

Investment in testes does not appear to trade off against investment in male-male contest behavior. This result may mean that neither contest behavior nor testes investment are energetically demanding enough to generate a tradeoff in this species. While some studies have shown that contest behavior is costly (see Introduction), in

general there has been little quantification of male reproductive investment into intrasexual contest behavior (Hoglund and Sheldon 1998, Kotiaho and Simmons 2003), and there have been few attempts to examine how such investment might limit the opportunity for investment into postcopulatory traits such as testes size. Repeating the present study under conditions of nutrient restriction may shed some light onto the question of the costs of such investment, and their interaction. The current study was conducted with *ad libitum* access to food. Under such conditions, tradeoffs that might occur in nature, where food is limited, might not be observed (Roff 1992, Stearns 1992).

We can infer from Experiment 3 that testes size adjustment is not simply due to an effect of mating on testes size. In other words, the small mean testes size observed in female-biased ratios is not a consequence of sperm depletion caused by elevated mating rates in female-biased cups. Similar results have been reported for stalk-eyed flies, in that mating frequency does not influence testes size (Rogers et al. 2005). However, the proximate mechanisms by which testes size is adjusted are unknown. It is possible that newly-eclosing males directly assess operational sex ratio via interactions with other males and females, and regulate testes development accordingly. Alternatively, our results may reflect a physiological tradeoff between investment in testes and investment in mating-related activities such as courtship, as males in female-biased cups spend relatively more time courting and less time engaging in contests with other males than do males in male-biased cups. Indeed, when courtship is broken out from other mating effort in our results from Experiment 2, courtship increases as the sex ratio becomes more female biased. However, Experiment 3, which manipulated the occurrence of mating, did

not necessarily manipulate the occurrence of courtship. It would be interesting to manipulate courtship independently of OSR and observe the effect on testes, perhaps through hormonal treatment.

In closing, it is worth noting that some studies have failed to find an intraspecific association between social environment and testes size (e.g. Harris & Moore 2004), suggesting that conditions favoring testes size adjustment may not always be met. Interestingly, Harris and Moore's study of cockroaches, *Nauphoeta cinerea*, did find a positive relationship between presence of rivals and spermatophore size. Since large spermatophores inhibit female remating in this species, their results imply that male cockroaches respond to social environment by investing in traits that minimize 'head to head' competition among sperm (a defensive strategy), rather than traits that make interacting sperm more directly competitive (an offensive strategy). Similarly, species such as *Drosophila melanogaster* reduce levels of sperm competition by transferring accessory gland products during mating that suppress female remating (Wolfner 2002). Traits that alleviate sperm competition will reduce the intensity of sexual selection for traits that improve a male's performance in sperm competition; thus, investment in larger testes under intense sperm competition may not be favored in species that evolve effective avoidance mechanisms. Future studies of intraspecific adjustments to male gonads in response to social environment may therefore benefit from a consideration of whether a given species is likely to invest in traits that reduce sperm competition or in traits that make a male's ejaculations more competitive.

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Figure Legends

Figure 1. The relationship between mean testes area and mid-wing vein length, an estimator of body size, in Experiment 1. Larger males tend to have larger testes (Linear mixed-effects model fit by REML; Effects test for body size: $F_{1,85} = 80.2$, $p < 0.0001$).

Figure 2. The pattern in mean testes area over sex ratio treatments before taking body size into account (Experiment 1). Testes size varied significantly among treatments, with significance due largely to the large mean testes area in 9M:3F treatment and the small mean testes area in the 1M:11F treatment (ANOVA; $F_{4,116} = 3.68$, $p = 0.007$). Error bars are SE.

Figure 3. The relationship between mean testes area and sex ratio treatment after taking body size into account (Experiment 1). The log of residual mean testis area (generated by removing body size) is plotted against number of males. Number of males is used instead of sex ratio because it contains the same information, but allows regression of variables. Variation in mean testes area is explained significantly by sex ratio treatment, with males in relatively more male-biased ratios having relatively larger testes (ANOVA; $F_{4,116} = 5.53$, $p = 0.0004$). Error bars are SE.

Figure 4. The relationship between per capita contest behavior between males and sex ratio treatment (Experiment 2). Overall, the frequency of contest behavior varies

significantly among sex ratio treatments (ANOVA; Effects test for sex ratio $F_{3,240} = 6.41$, $p = 0.0003$). Error bars are SE.

Figure 5. The relationship between per capita male mating effort and operational sex ratio. (Experiment 2; ANOVA; Effects test for sex ratio $F_{3,240} = 37.8$, $p < 0.0001$). Error bars are SE.

Figure 6. The relationship between mean testes area and mid-wing vein length, an estimator of body size, in Experiment 3. Larger males tend to have larger testes (ANOVA; Effects test for body size: $F_{1,43} = 6.89$, $p = 0.01$).

Figure 7. The relationship between opportunity to mate and testes size (Experiment 3). Testes size did not differ between treatments 2:10 M (female-biased sex ratio, mating allowed) and 2:10 NM (female-biased sex ratio, mating prevented), while testes size in treatments 9:3 (male biased sex ratio, mating prevented) differed from both of these treatments. (ANOVA; Effects test for group: $F_{2,43} = 8.26$, $p = 0.0009$). Error bars are SE.

Figure 1.

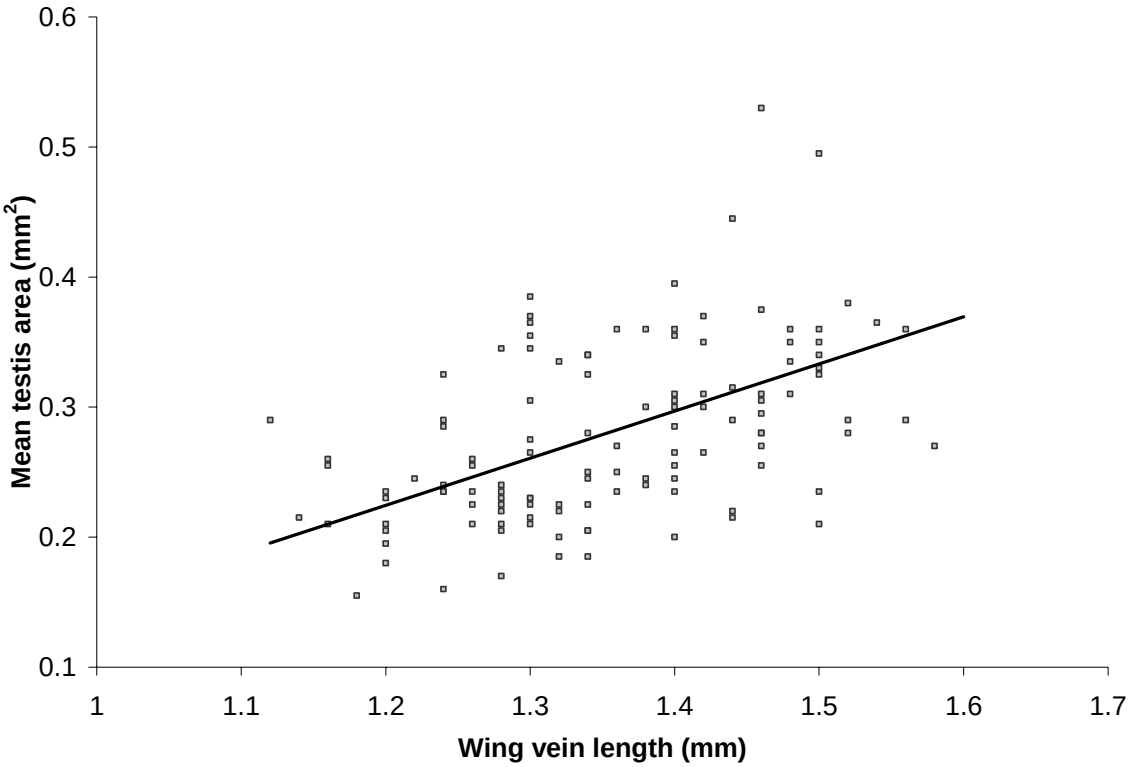

0.6
0.5
0.4
0.3
0.2
0.1
Mean testis area (mm2)
1
1.1
1.2
1.3
1.4
1.5
1.6
1.7
Wing vein length (mm)

Figure 2.

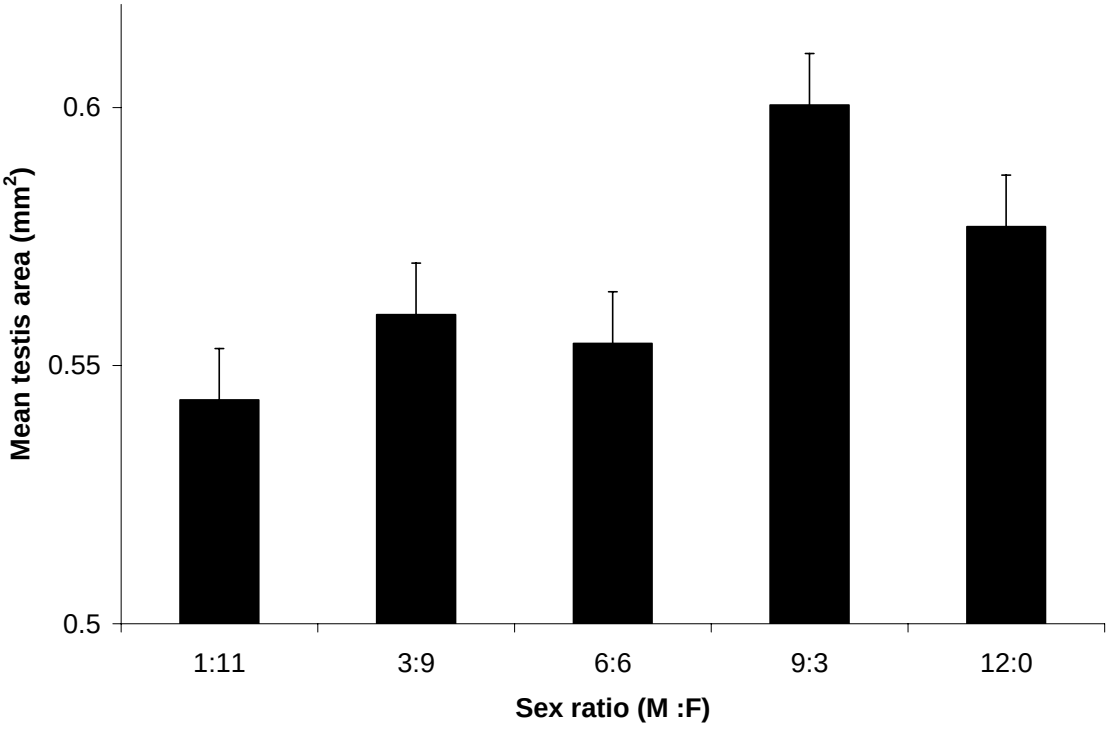


Figure 3.

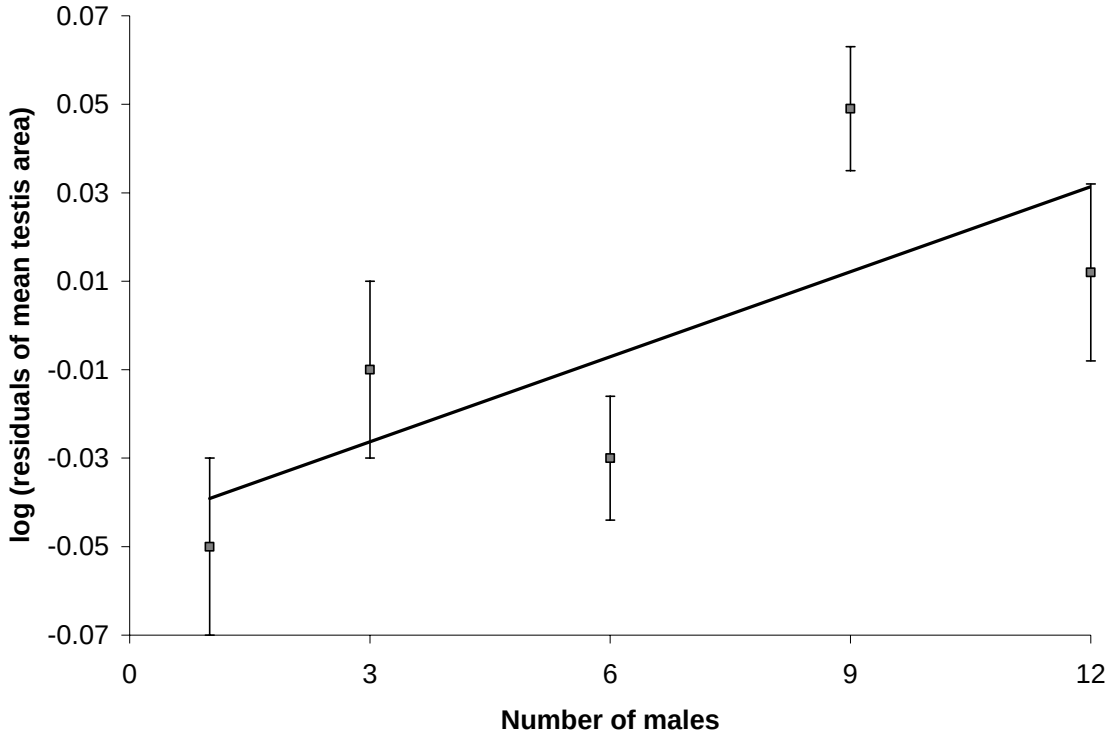

0.07
0.05
0.03
0.01
-0.01
-0.03
-0.05
-0.07
log (residuals of mean testis area)
0
3
6
9
12
Number of males

Figure 4.

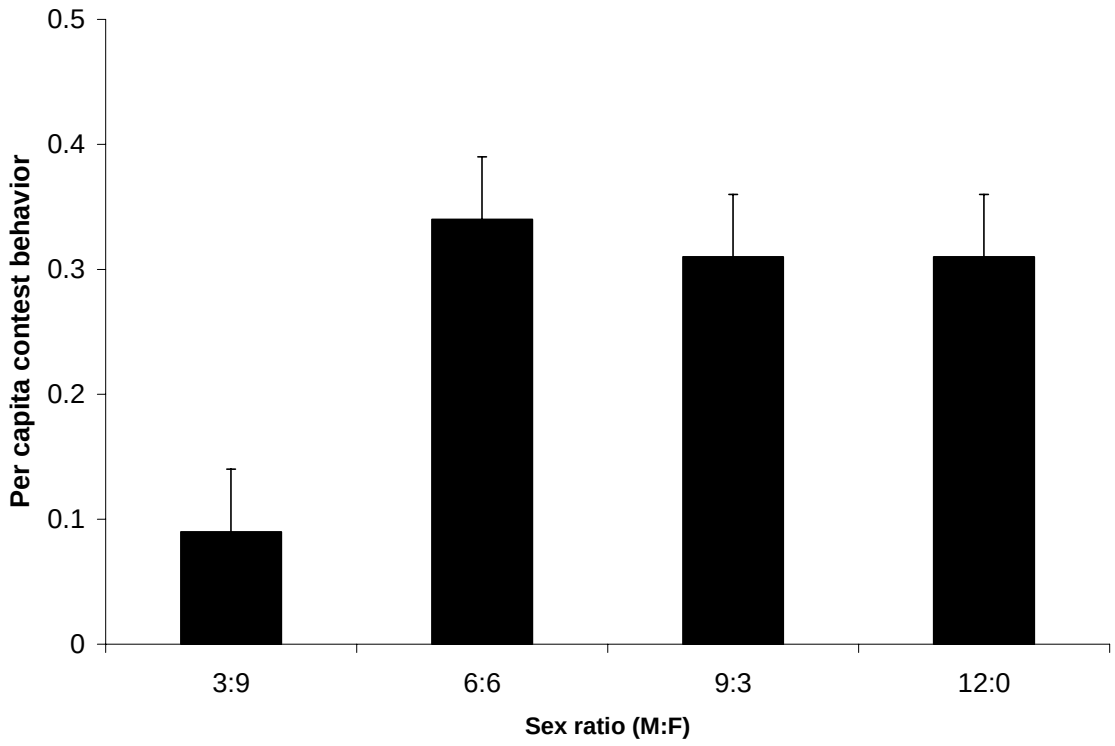

0.5
0.4
0.3
0.2
0.1
0
Per capita contest behavior
3:9
6:6
9:3
12:0
Sex ratio (M:F)

Figure 5.

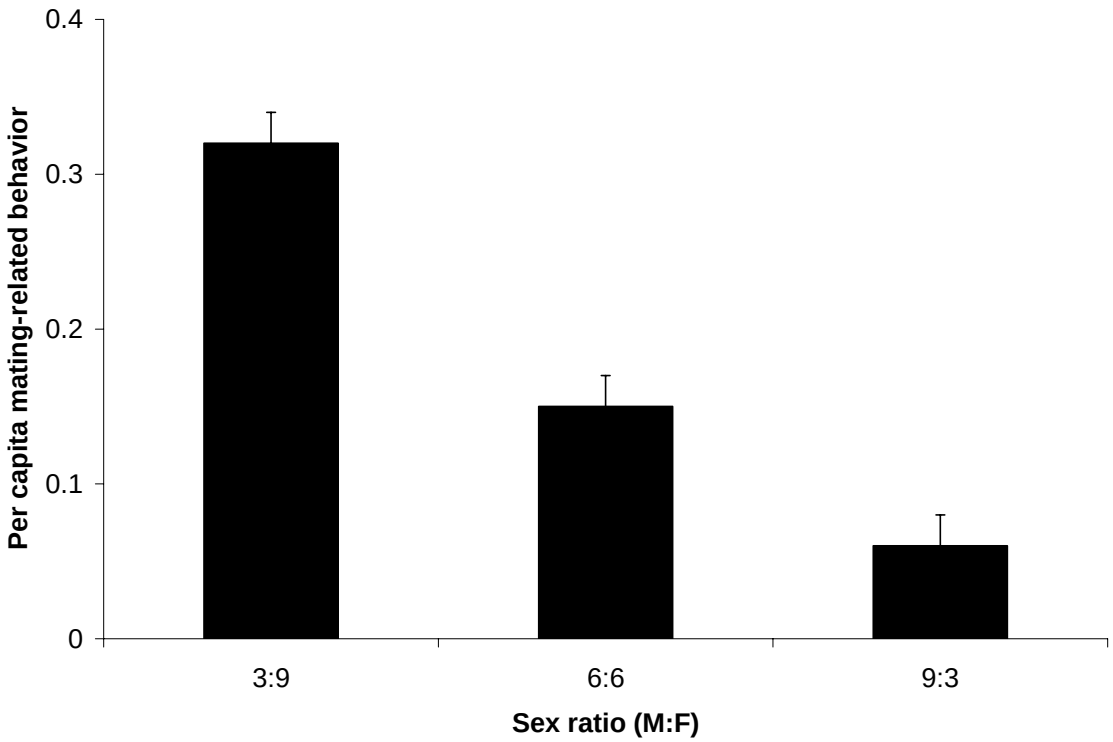

0.4
0.3
0.2
0.1
0
Per capita mating-related behavior
3:9
6:6
9:3
Sex ratio (M:F)

Figure 6.

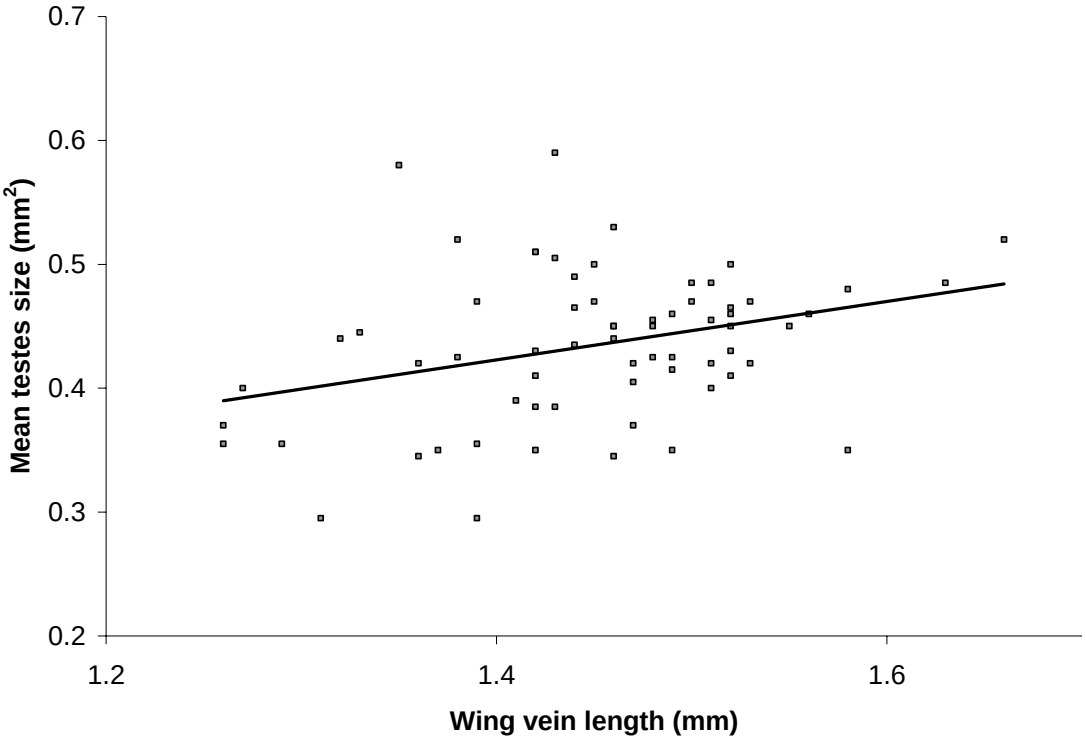
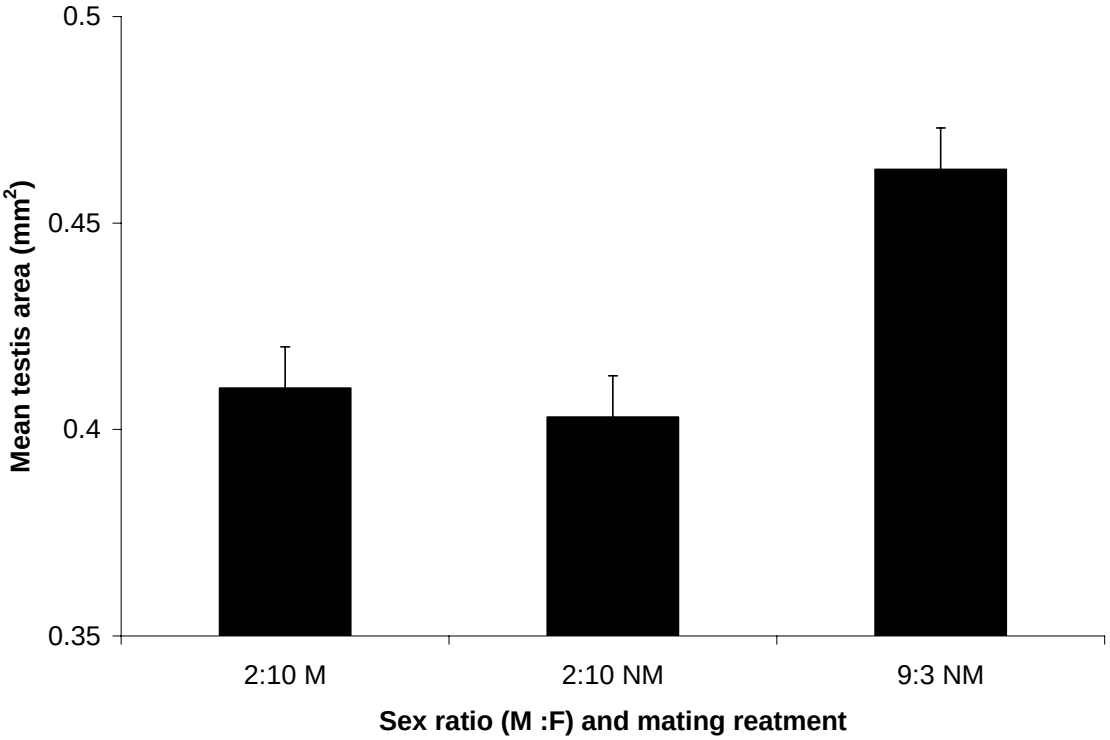

0.7
0.6
0.5
0.4
0.3
0.2
Mean testes size (mm2)
1.2
1.4
1.6
Wing vein length (mm)

Figure 7.


0.5
0.45
0.4
0.35
Mean testis area (mm2)
2:10 M
2:10 NM
9:3 NM
Sex ratio (M :F) and mating reatment

APPENDIX C

RESOURCE ALLOCATION TO TESTES IN WALNUT FLIES: DIVERSE ALLOCATION STRATEGIES FOR CARBON AND NITROGEN

Resource allocation to testes in walnut flies: diverse allocation strategies for carbon and nitrogen

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Abstract

Testes size often predicts whether a male will win during episodes of sperm competition. However, little is known about the source of nutrients allocated to testes development, or testes plasticity under varying nutrient availability. Among many holometabolous insects, metabolic resources can be derived from the larval or the adult diet. Distinguishing the source of nutrients allocated to testes can shed light on life history factors that shape the evolution of male reproductive strategies. For instance, if allocated resources are derived mostly from larval reserves, then maternal influences such as oviposition decisions may have a strong influence on male reproductive success. Conversely, if allocated resources are mostly derived from adult stores, then male reproductive success may depend more on the foraging ability of that individual. Here we used an experimental approach to assess resource allocation to testes development in walnut flies (*Rhagoletis juglandis*) from differing nutritional backgrounds. We fed adult male walnut flies on sugar and yeast diets that contrasted with the larval diet in carbon and nitrogen stable isotope ratios. This design allowed us to assess the dietary source of testes carbon and nitrogen and its change over time under differing adult nutritional regimes. We found significant replacement of larval carbon with adult carbon sources, but almost no replacement of larval nitrogen with adult nitrogen sources. These results imply that, instead of possessing a single allocation strategy, walnut flies are capital breeders for nitrogen (relying on larval reserves), and income breeders for carbon (relying more on adult resources).

Key words: resource allocation; sperm competition; stable isotope; insects; capital breeding

Introduction

Sperm competition is an important selection pressure on male traits in many species (Simmons 2001; Birkhead and Møller 1998). Males that experience intense sperm competition often allocate more resources towards traits such as large testes and large ejaculates than do males experiencing low levels of sperm competition (Pitcher *et al*. 2005; Schulte-Hostedde & Millar 2004; Preston *et al*. 2003; Hosken *et al*. 2001; Stockley *et al*. 1997; Gage 1994; Møller 1991; Svärd & Wiklund 1989). Larger testes and larger ejaculates necessarily require more resources to produce. Thus, resource availability can have important consequences for the development of traits such as testes size. However, little is known about how and when nutritional resources are allocated to testes development. Among species that use distinct resources across different life stages, knowledge about which life stage is involved in the acquisition of resources for testes development can shed light on factors that shape the evolution of male reproductive strategies.

Holometabolous insects (those that undergo complete metamorphosis) are useful models for exploring allocation decisions, as larval and adult diets often differ in nutritional composition and availability to specific tissues (Boggs 1981; Zera and Harshman 2001; O'Brien et al. 2002). Insects can vary in the degree to which they rely on larval reserves or adult feeding, ranging from a "capital" strategy, involving reliance mainly on larval reserves, to more of an "income" strategy, involving reliance mainly on adult feeding (Stearns 1992, Jönsson 1997). Determining whether testes tissue derives mostly from adult or larval stores has important implications for selection pressures that

may shape testes size. If an animal is primarily a capital breeder with respect to testes, then testes size may depend on the quality of the larval diet. In turn, larval diet quality is heavily influenced by where the mother laid her eggs (Thompson 1988; Mayhew 1997; Janz 2005; Fontellas-Brandalha and Zucoloto 2004; Digweed 2006); thus, testes size may ultimately be influenced by maternal effects. Conversely, if an animal is mostly an income breeder with respect to testes, then testes size may depend on adult foraging ability or resource availability. In sum, the reproductive success of a male might be profoundly influenced by nutritional constraints in either the larval or the adult life stages.

Here, we examine which life stage contributes the nutrients allocated to testes in *Rhagoletis juglandis*, the walnut fly (Diptera: Tephritidae). Like other members of this temperate genus, this species is characterized by a resource defense mating system, where males engage in contests and monopolize fruit, thereby gaining access to females who come to the fruit to oviposit (Papaj 1994). Both males and females mate multiply (Nufio et al. 2000), and as in other polyandrous species (e.g. Gage 1994, Stockley et al. 1997), testes size is relatively large in proportion to body size. There is also evidence that males adjust testes size in relation to perceived levels of sperm competition (Carsten-Conner and Papaj, in review). Taken together, these factors suggest that allocation of resources to testes could be an important component of overall reproductive strategy for male walnut flies.

If allocating resources to testes is important for reproductive success, one might expect that males would invest a large proportion of both larval and adult resources to

testes. However, investment constraints might arise through nutritional constraints imposed by diet quality. Among walnut flies, a poor-quality larval environment (small fruit size or increased larval density) leads to small body size (Nufio and Papaj 2004), illustrating that overall resource availability is reduced under these conditions. Thus, we predicted that small flies would experience more investment constraints on capital reserves than would large flies, leading to reliance on an income strategy for testes investment. There are reasons to expect that small males will direct their income resources to testes rather than other reproductive activities. Large walnut flies outcompete small flies in contests (Papaj, unpublished data), and small males gain fewer mates than do large males (Carsten-Conner, unpublished data). If a small male is unlikely to win contests during pre-copulatory competition, it may be to his advantage to invest maximally in testes in order to try to compete in the post-copulatory arena. Large males, which eclose from pupation with a reproductive advantage, may have less need to direct income resources to testes, freeing up these resources for allocation to other functions. Here, we ask whether small flies can compensate for their size by allocating relatively more adult resources to testes than do large flies.

Methods

We tested our predictions by feeding adult walnut flies isotopically contrasting diets, in order to determine the proportion of testes carbon and nitrogen deriving from the larval vs. the adult lifestage. In order to trace larval vs. adult sources of tissue carbon, we took advantage of the naturally occurring ^{13}C enrichment of C_4 plants relative to C_3 plants

(O'Leary 1988). The larval diet consists of the fruit of a C_3 plant, walnut (*Juglans major*). Adults in the lab were maintained on cane sugar (a C_4 plant) and yeast. We used two alternate batches of yeast, grown under identical conditions on cane sugar (C_4) and beet sugar (C_3), in order to distinguish yeast-derived carbon from sugar-derived carbon. Thus, each group of flies was grown on an isotopically unique combination of larval and adult carbon sources. In order to trace larval vs. adult sources of tissue nitrogen we differentially labeled the two yeast forms using ^{15}N labeled ammonium sulfate in the growth medium to provide a contrast with walnut fruit ^{15}N. The isotope signatures of all dietary components are given in Table 1.

We used these isotopic differences to calculate the proportional contribution of larval and adult sources of carbon and nitrogen to testes. In order to evaluate whether allocation of larval vs. adult resources to testes was different with respect to the rest of the fly, we also estimated these values for thoraxes.

Experimental protocol

Flies were collected as pupae in southern Arizona during the summer of 2005. Collections consist of thoroughly mixed and randomized pupae from multiple fruits and trees, which minimizes relatedness between individuals in a given collection cup. Pupae were maintained at 4^{o}C prior to the study, and warmed to room temperature (ca. 29^{o}C) until eclosion approximately 3 weeks later. Within 48 hours of eclosion, we aspirated male flies into 12 clear plastic 473-ml cups. We made visual estimates of body size and placed 10 small flies into six of the cups, and 10 large flies into the other six cups. We

only used flies that appeared to lie on the small and large ends of a continuum of body size—that is, medium-size flies were avoided. We supplied the cups with an ad libitum amount of one of two types of yeast: half the cups received yeast enriched in ^{15}N and the other half received unenriched yeast. These yeasts were provided by M. Tatar, developed as described in O'Brien et al. (in review).

The flies were reared in an incubator kept at ca. 27 ^{o}C. One cup containing small flies and one cup containing large flies were collected and frozen at three different time intervals: 5 days, 10 days, and 15 days post eclosion. We also harvested 10 large and 10 small flies at 0 days post eclosion in order to obtain a baseline estimate for larval reserves. This entire experiment was replicated three times.

For each cup in each replicate, we dissected out the testes of each fly under a dissecting scope and placed all fly testes from a single cup into a pre-weighed tin capsule. Samples were oven dried at 60 ^{o}C for over 24 hours, and dry mass was recorded. Pooling the testes in this manner was necessary, as testes weight from a single fly was too small to obtain an accurate isotopic analysis. We also photographed the wings of each fly in order to confirm body size, as mid-wing vein length correlates well with body mass (H. Alonso-Pimentel, unpublished data), and we photographed testes to estimate testes area. Mid-wing vein length and testes area were calculated using ImageJ software (National Institutes of Health, version 1.32j). For estimates of thorax allocation, we used a subset of the animals in each cup for isotope analysis. Because thorax weight greatly exceeded testes weight, all animals from a cup could not be pooled into a single sample. Legs and

wings were removed from all thoraxes, and we randomly selected thoraxes from each cup for analysis.

Determination of stable isotope ratios

The carbon and nitrogen isotope ratios of testes and thoraxes were measured at the Alaska Stable Isotope Facility using continuous flow isotope ratio mass spectrometry. A Costech ECS4010 Elemental Analyzer was used to combust samples to CO_2 and N_2 gases. The gases were fed to a Finnigan Delta Plus XP isotope ratio mass spectrometer, where the gases were ionized and separated by a magnetic field (e.g. $^{12}CO_2$ and $^{13}CO_2$). Data are expressed in delta notation as $((R_{sample}/R_{standard})-1 \times 1000$. R is the ratio of heavy to light isotope (for both carbon and nitrogen) and standards are Vienna PDB for carbon and Air N for nitrogen. We obtained C/N ratios, $\delta^{13}C$, and $\delta^{15}N$ values. We concurrently weighed and ran multiple peptone standards to assess analytical precision; these gave values of $\delta^{13}C = -15.84 \pm 0.07$ ‰ (SD), and $\delta^{15}N = 7.01 \pm 0.22$ ‰ (SD).

Calculations

The isotope ratio of nitrogen or carbon in tissue (% N, % C) is a function of the isotopic signatures of its nitrogen or carbon sources weighted by their proportional contributions to the tissue. These values might be slightly offset by slight shifts in isotope ratio (fractionation) that occur during assimilation and metabolism of nutrients (e.g. Spence and Rosenheim 2005).

Carbon in the testes or thorax could be derived from three sources: walnut fruit in the larval stage ($\%C_{fruit}$), and both yeast ($\%C_{yeast}$) and sugar ($\%C_{sugar}$) in the adult stage:

$$\delta^{13}C_{tissue} = \delta^{13}C_{fruit}(\%C_{fruit}) + \delta^{13}C_{yeast}(\%C_{yeast}) + \delta^{13}C_{sugar}(\%C_{sugar}) + \Delta$$

$$1 = \%C_{fruit} + \%C_{yeast} + \%C_{sugar}$$

Where Δ describes the isotopic offset between diet and tissue. We assume that the actual Δ value lies between -1 ‰ to +1 ‰, which is within the standard range for herbivorous insects (Spence & Rosenheim 2005), and we solve for $\Delta = 0, -1, \& +1$. $\Delta = 0$ is represented graphically.

We first calculated the percent C derived from yeast, and then used that value to calculate the carbon contributions of fruit (obtained at larval stage) and sugar (obtained at adult stage).

We calculated percent C derived from yeast as follows:

$$\%C \text{ from yeast} = (\delta^{13}C_{labeled\ tissue} - \delta^{13}C_{unlabeled\ tissue}) / (\delta^{13}C_{labeled\ yeast} - \delta^{13}C_{unlabeled\ yeast})$$

We calculated the amount of nitrogen in the testes and thorax derived from adult (yeast) sources by using the following equation:

$$\%N_{yeast} = (\delta^{15}N_{labeled\ tissue} - \delta^{15}N_{unlabeled\ tissue}) / (\delta^{15}N_{labeled\ yeast} - \delta^{15}N_{unlabeled\ yeast}),$$

where $\delta^{15}N_{labeled\ tissue}$ is the isotopic ratio of nitrogen from males fed labeled yeast, $\delta^{15}N_{unlabeled\ tissue}$ is the isotopic ratio of nitrogen from males fed unlabeled yeast, and $\delta^{15}N_{labeled\ yeast}$ and $\delta^{15}N_{unlabeled\ yeast}$ are the actual isotopic ratios of the nitrogen in the two yeasts. Because all other nitrogen must be derived from larval sources, the percent nitrogen derived from walnut fruit was simply = 100 - $\%N_{yeast}$.

We calculated the change in carbon in testes and thorax over time using the following turnover model:

$$\%C_{(day)} = 1\text{-}e^{-r * day} (\%C_f - \%\ C_i) + \%\ C_i,$$

where $\%C_i$ is the initial %C, C_f is the final %C, and r is the fractional turnover rate (O'Brien et al. 2000, 2002, 2004, Min et al. 2006). We used a nonlinear model to estimate r, C_i, and C_f. The value t_{50}, time to 50% of maximal turnover, was also calculated ($t_{50} = \ln(2)/r$). We fit this model to data on small and large flies separately, as well as to the pooled data, and compared the fit between the pooled and separate data to determine if there were differences in parameters between small and large flies (Motulsky and Ransnas 1987). The nonlinear model was a poor fit for the nitrogen data; thus, we used a linear model to ask whether age or size predicted % adult or larval nitrogen in testes and thorax.

We also compared C:N ratios in thorax and testes over time, and used ANOVA to ask whether age, size, or age*size predicted overall % adult carbon and % adult nitrogen in the testes or thorax. Finally, we calculated the total mass of carbon and nitrogen invested in both testes and thorax in grams from all dietary sources by multiplying tissue dry mass x %C or %N divided by 100.

In order to confirm previous data indicating that testes grow continuously until ca. 11 days in these flies (Carsten-Conner, unpublished data), we used a linear model to ask whether testes area was predicted by age and body size. We also fit testes size data over time to a nonlinear model in order to determine if there were differences in growth rate between small and large flies. We used the turnover model described above to fit the testes growth data. The model fits the general shape of growth data in the same manner as turnover data; thus, the model was appropriate for testes growth data. All statistical analyses were carried out using JMP-IN statistical software (SAS Inc.; Cary, North Carolina, version 5.1.2). Results are reported with standard errors rather than standard deviations.

Results

Body size and testes growth

In this study, we visually categorized flies at eclosion as large versus small, and after flies were frozen, used mid-wing vein length to quantify differences in body size. The mean mid-wing vein length measurement for all flies was 1.4 ± 0.01 mm. The mean vein length of small versus large flies was highly significantly different (mean ± SE for small = 1.27

± 0.007 mm, mean ± SE for large = 1.56 ± 0.006 mm; t-test, t = 27.9, df = 347, p< 0.0001), although there was a slight overlap in mid-wing vein length between size classes (27 out of 350 flies overlapped).

Measurements of testes area over time corroborated previous results (Carsten-Conner unpublished data) that testes continue to grow until approximately 11 days after eclosion (Fig. 1). We also fitted nonlinear models to testes area over time to describe the differences in testes growth rate between small and large flies (Fig. 1). The results indicate that there were differences between large and small flies in initial (small C_i = 0.24 ± 0.01 mm, large C_i = 0.37 ± 0.01 mm) and final (small C_f = 0.34 ± 0.01 mm, large C_f = 0.49 ± 0.01mm) testes area, but not in growth rate (small r = 0.30 ± 0.13, large r = 0.21 ± 0.06).

C:N ratios, total %C and % N

The mean C:N ratio for testes was 4.16 ± 0.10 %. This ratio did not change over time. There were no differences between total % C or % N in testes by age, size, or age*size. The mean C:N ratio for thoraxes was 4.36 ± 0.30%. In contrast to testes, the C:N ratio increased over time, starting at 3.9 ± 0.07 % at day 0, and reaching 4.5 ± 0.07 % by day 5 (ANOVA, $F_{3,71}$ = 12.78, p< 0.001). There were no differences in C:N ratio between days 5, 10, or 15. The change in C:N ratio between day 0 and day 5 appears to be caused by a statistically significant decrease in nitrogen in the thorax, rather than an increase in carbon (mean N at day 0 for large flies = 12.3%, mean N at day 5 = 11.18%; effects test from ANOVA $F_{3,67}$ = 9.86, p < 0.001). There were also significant

differences in overall % N in thorax by size (effects test from ANOVA $F_{3,67} = 10.8$, $p = 0.0016$) and age*size (effects test from ANOVA $F_{3,67} = 3.2$, $p = 0.03$). Large flies had a higher percentage of nitrogen in thorax overall compared to small flies, largely driven by a higher percent of N at day 0 for large flies (12.9%), after which nitrogen level drops in large flies. This indicates that large, but not small flies, lose some amount of nitrogen from the thorax after eclosion that is not replaced. However, thorax weight remained constant over time.

Allocation of carbon to testes and thorax

The amount of carbon contributed to testes and thorax from yeast was not distinguishable from zero; thus, we calculated contributions from larval and adult sources by the following equation:

$$\%C \text{ from fruit} = ((\delta^{13}C_{tissue} - \Delta) - \delta^{13}C_{sugar})/ (\delta^{13}C_{fruit} - \delta^{13}C_{sugar}),$$

where $\delta^{13}C_{tissue}$ is the isotopic ratio for carbon from males, $\delta^{13}C_{sugar}$ is the isotopic ratio of sugar carbon and $\delta^{13}C_{fruit}$ is the isotopic ratio for walnut fruit (Again, Δ describes the isotopic offset between diet and tissue. We assume that the actual Δ value lies between -1 ‰ to +1 ‰). All signatures from labeled and unlabeled flies are reported in Table 2.

Carbon turned over rapidly in testes, with carbon from adult sugar constituting 52% of all testes C by day 5 (Fig. 2). Replacement of larval carbon continued at a slower rate until day 10. Day 15 was no different than day 10 for % adult dietary carbon in

testes. Because testes grow until approximately 11 days after eclosion, this pattern indicates that adult carbon is important in testes growth, but that carbon turnover largely stops once testes have reached maximal size. Adult carbon contribution to testes plateaus at about 68%; this value assumes a fractionation of 0 ‰. Isotopic fractionation for carbon was not calculated in this study; however, values generally range from 0 to -1% in herbivorous insects (Spence & Rosenheim 2005). If we assume a fractionation of ± 1 ‰, the percent of carbon from adult sugar in testes at plateau ranges from 61% to 75%. From the range of values, it is clear that flies incorporate a large amount of carbon from adult diet into their testes.

The turnover model yielded a fractional turnover rate of 0.28 ± 1.84 for adult carbon vs. time, and 0.28 ± 0.03 for larval carbon over time. This translates into a half life (time to 50% of maximal turnover) of 2.5 days. Although age significantly predicted carbon turnover, there were no differences in carbon turnover by size. The nonlinear model fitting carbon turnover by age and size fit the pooled data better than the separate data (Extra sum of squares test, $F_{3,17} = 0.133$, $p = 0.93$).

Carbon turned over less rapidly in thorax than in testes, and there were differences between large and small flies. Carbon from adult sugar constituted only 32% of all thorax C by day 5 for large flies, and 41% for small flies (Fig. 3). Replacement of larval carbon continued at a slower rate until day 15 for both sizes, when carbon from adult sugar reached 41% of all thorax C for large flies, and 48% for small flies. Again, these values assume a fractionation of 0 ‰. If fractionation was + 1 ‰, day 15 values would be 48% for large flies and 59% for small flies. Assuming a fractionation of -1 ‰ shifts these

values to 34% for large flies, and 40% for small flies. However, while absolute values might vary depending on fractionation, the difference between large and small flies would remain constant.

In contrast to testes, there were differences in fractional carbon turnover rates by size (nonlinear model results: $F_{3,34} = 5.66$, $p = 0.003$), with small flies turning over carbon more rapidly than large flies (small $r = 0.43 \pm 0.06$, large $r = 0.28 \pm 0.07$ for adult carbon). This translates into a half life (time to 50% of maximal turnover) of 1.6 days for small flies, and 2.5 for large flies.

Allocation of nitrogen to testes and thorax

In contrast to carbon, flies used little adult nitrogen overall in testes (Fig. 4). Nitrogen contribution to testes from yeast was not significantly different from zero until day 15, when the % adult nitrogen in testes rose to 7% of overall testes nitrogen. Because our data indicate that testes growth tapers off between 10 and 15 days, this late turnover suggests that animals are not using this nitrogen to increase testes size, but rather for some aspect of sperm production that is independent of testes size. That is, rather than incorporating this nitrogen directly into testes tissue, perhaps nitrogen is used directly in maturing larger numbers of sperm, or incorporated directly into sperm tissue in some other fashion. The data were poorly fit by an exponential turnover model; thus, we used a linear model to predict whether age, size, or age* size affected % adult nitrogen in testes. Although there was an effect of age on testes nitrogen from adult diet, as described above, there was no effect of size or age*size (Table 3).

However, as with testes, flies used little adult nitrogen overall in thorax tissue. The linear model asking whether size, age, or age* size predicts nitrogen contribution to thorax showed that age is significant in predicting % adult nitrogen in thorax (effects test from ANOVA $F_{1,36} = 2.78$, $p = 0.057$). While age is a significant predictor, contribution from adult yeast to thorax rose to only 2.6% by day 15 (Fig. 5). There was also an effect of size, but not age*size, on adult nitrogen contribution to thorax (Table 4). Small flies averaged 2.16 ± 0.45 % adult nitrogen in thorax, while large flies averaged 0.43 ± 0.43 % (Fig. 6).

Relative allocation to each tissue

The relative allocation of resources to testes vs. thorax for large vs. small flies can be assessed in two ways: first, comparing the ratio of thorax weight to testes weight for each size class can reveal differences in total investment to each structure, and second, comparing the ratio of adult to larval investment (in grams C or N) in each structure for each size class can reveal differences in relative allocation of carbon and nitrogen.

In terms of total investment in each structure, the ratio of thorax: testes weight (considering the weight of both testes) for small flies is 7.3, while it is 12.6 for large flies. Thorax weight is a very good proxy for total body weight; regressing dry thorax weight against dry body weight reveals an almost perfect correlation (L. Carsten-Conner, unpublished data, ANOVA, $F_{1,25} = 782.1$, $p < 0.0001$, $R^2 = 0.97$), with thorax constituting about 41% of total body weight. Thus, small flies allocate a higher percentage of total body weight to testes than to thorax.

We also assessed the ratio of adult: larval carbon (g) allocated to both testes and thorax for small and large flies. Small flies allocate more total adult carbon to thorax, and less total adult carbon to testes than do large flies (Figure 7). There were no differences in adult: larval nitrogen (g) allocated to testes vs. thorax for small vs. large flies.

Discussion

Overall allocation strategy

The relatively low carbon to nitrogen ratio in testes (see, e.g. Robbins et al. 2005 for various C:N ratios) indicates that testes growth is a nitrogen-demanding process, yet flies showed relatively little contribution of nitrogen from adult dietary yeast to testes growth, overall. This result is surprising, as other data show that males fed diets lacking in yeast (the only source of adult nitrogen in the laboratory diet) develop smaller testes than do flies with unlimited access to yeast (Carsten-Conner, unpublished data). It is possible that the flies were not eating the yeast provided in this study; however, three lines of evidence contradict that possibility. First, our results do show some incorporation of adult yeast into both thorax and testes tissue, so it appears that at least small amounts were ingested. Secondly, comparison of the present data to unpublished data (the study described above) reveals similarity in final testes size between flies in this study and flies that were fed yeast (in the earlier study, flies were fed hydrolyzed yeast rather than whole yeast). In the earlier study, flies that had access to yeast developed testes of 0.38 ± 0.01 mm^2, while in this study, they developed testes 0.41 ± 0.01 mm^2 in size. In contrast, flies without access to yeast developed significantly smaller testes (0.29 ± 0.01 mm^2). Body size was similar

between the two data sets (earlier study mid-wing vein = 1.32 ± 0.01 mm; present study mid-wing vein = 1.4 ± 0.01 mm). It seems very unlikely that flies in the present study could have attained such a large testes size without the ingestion of yeast. Third, in several earlier behavioral studies, anecdotal observations indicated that males spent copious time feeding on sugar, but little time feeding on yeast. Thus, it is likely that walnut flies do not generally ingest large amounts of yeast in the laboratory conditions provided, but that such ingestion still has a positive effect on testes growth.

Taken together, results are puzzling in that yeast ingestion appears to increase testes size, but there is little incorporation of adult nitrogen into testes in the present study. It is possible that flies need only trace amounts of nitrogen from adult dietary sources to develop their testes, but that these trace amounts have large effects on size. Alternatively, it is possible that nitrogen is not the limiting factor for testes growth. Yeast contains not only nitrogen, but also B-complex vitamins, which could be important in testes growth. Flies may ingest enough yeast to release a constraint on testes growth posed by a lack of B vitamins in the larval substrate, and not show evidence of nitrogen incorporation into testes from yeast. Finally, nitrogen ingested may be incorporated into other tissues, which in turn could cue testes maturation. For instance, if adult nitrogen were incorporated into accessory glands rather than testes, this could conceivably serve as a cue that prompts maturation of larger testes.

Assuming that flies are eating the laboratory yeast, the low incorporation of nitrogen from adult dietary sources into testes and thorax indicates that these flies are capital, rather than income, breeders with respect to nitrogen. In contrast, the flies appear

to be income breeders with respect to carbon. Testes carbon turned over rapidly, reaching a final concentration of around 65% carbon from adult sugar in testes tissue. Similarly, thorax tissue turned over to a final concentration of over 40% carbon from adult sugar. Most of the literature examining dietary life history strategy categorizes animals as capital vs. income breeders, without distinguishing between different components of resource (but see Casas et al. 2005). This study demonstrates that animals may differ dramatically in reproductive strategy from one resource currency to another. Our results underscore the fact that nutrients have different functions within the animal, and are not necessarily interchangeable. Carbon, for instance, is used primarily for energy metabolism, while nitrogen is critically important in biosynthesis. A consideration of what stage each nutrient class derives from can shed important light on many aspects of an animal's life history strategy, linking resource availability with activities throughout its lifespan. For instance, Kemp and Alcock (2003) predict that contest behavior should be profoundly affected by breeding strategy. Contest behavior can be energetically costly (Marden and Waage 1990; Parker and Thompson 1980), and species with a capital breeding strategy cannot supplement larval reserves with adult feeding to fuel contests. Because dipterans primarily use sugars to fuel energy metabolism and flight rather than breaking down stored lipids (Candy 1989), expensive activities such as contests likely require income carbon. This prediction is consistent with our results in that male walnut flies, which engage extensively in contests, are income breeders for carbon.

In terms of nitrogen, we might use our results to predict the distribution of nitrogenous resources in the adult environment. In particular, a capital breeding strategy

for nitrogen is consistent with the idea that nitrogenous resources are more patchy and ephemeral than carbon resources in the adult environment. While relatively little is known about adult feeding strategies for walnut flies, flies in the genus *Rhagoletis* are thought to obtain nitrogen primarily from bird feces deposited on foliage (Prokopy and Papaj 2000). It is likely that bird feces are relatively less abundant than foliar and fruit leachates, the primary sources of adult carbon. If adult nitrogenous resources are hard to locate, than reliance on stored larval reserves of nitrogen to aid in biosynthesis of new testes tissue would be a good strategy.

Allocation strategy with respect to body size

We initially predicted that small flies should rely more on an income strategy than large flies with respect to testes investment. Large flies are at a distinct reproductive advantage in terms of absolute testes size, ability to gain mates, and ability to win contests; thus, we predicted that small flies might attempt to compensate for their disadvantages by using more of their adult resources towards testes growth than would large flies. Contrary to this prediction, there were no differences in carbon or nitrogen turnover (larval to adult) in testes tissue between small and large flies. However, our results for ratio of thorax: testes weight show that small flies allocate a higher total percentage of body weight to testes than do large flies. Because testes growth rate is not different between large and small flies, and small flies are not turning over carbon or nitrogen in testes more rapidly than large flies, the relatively larger testes size of small flies appears to be established during the larval stage. Indeed, we found that small flies allocate relatively more adult

resources to thorax than do large flies. Thus, small flies may "make up" for their size-imposed disadvantages by allocating more to testes during the larval stage, rather than in the adult stage.

Implications for the evolution of male reproductive strategies

Nitrogenous resources are critical in biosynthesis of tissue, and walnut flies appear to be capital breeders with respect to nitrogen. Thus, fully developed testes size likely depends in large part on the larval environment, although adult nutrition also plays a role. Therefore, to the extent that increased testes size translates into a postcopulatory advantage as it does in other species, larval nutrition is an important determinant of sperm competitive ability in males. Thus, testes size may ultimately depend more on maternal oviposition decisions than on adult foraging success or resource environment. Mothers that lay their eggs in larger fruit will have larger offspring with larger *absolute* testes size. Mothers that lay their eggs in smaller fruit will have smaller offspring with *relatively* larger testes for their body size. Nufio and Papaj (2004) found that females prefer to oviposit in large fruit, which has an overall positive effect on larval size and fecundity. However, females also have a strong preference to reuse hosts, which generates larval competition and has an overall negative effect on size and fecundity. Nufio and Papaj interpreted this to mean that there is direct selection on females to reduce oviposition-related costs and maximize number, rather than quality, of offspring. The present results suggest that small flies possess some compensatory mechanisms in

terms of testes size; thus, an oviposition decision that results in decreased offspring size may be less detrimental to offspring sperm competitive ability than predicted.

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Table 1. Experimentally determined values for carbon and nitrogen signatures of different diet components. Data are given with standard deviations.

Source	$\delta^{13}C$	$\delta^{15}N$
Sugar cane	-11.96 ± 0.09	N/A
Walnut fruit	-25.65 ± 0.47	1.46 ± 2.23
Enriched yeast	-10.12 ± 0.04	252.54 ± 1.58
Unenriched yeast	-23.95 ± 0.05	-3.78 ± 0.05

Table 2. Isotopic signatures of tissues in this study. Data are expressed in delta notation as $((R_{sample}/R_{standard})-1) \times 1000$. R is the ratio of heavy to light isotope (for both carbon and nitrogen) and standards are Vienna PDB for carbon and Atmospheric N for nitrogen. L = labeled yeast, U = unlabeled yeast. Data are given with standard errors.

	Nitrogen ($\delta^{15}N$)		**Carbon ($\delta^{13}C$)**	
Yeast	L	U	L	U
Age (days)	**Testes**			
0		6.7 ± 0.32		-23.54 ± 0.16
5	7.61 ± 0.82	5.63 ± 0.53	-18.46 ± 0.41	-18.49 ± 0.32
10	8.12 ± 1.17	5.78 ± 0.25	-17.75 ± 0.32	-17.09 ± 0.29
15	23.07 ± 4.0	4.98 ± 0.39	-16.40 ± 0.2	-16.28 ± 0.22
	Thorax			
0		7.83 ± 0.24		-23.86 ± 0.18
5	10.22 ± 0.68	7.37 ± 0.36	-20.54 ± 0.19	-20.63 ± 0.29
10	10.14 ± 0.95	8.64 ± 0.44	-20.07 ± 0.17	-19.96 ± 0.21
15	15.24 ± 2.56	8.43 ± 0.29	-19.85 ± 0.18	-19.58 ± 0.24

Table 3. Age significantly predicts % adult nitrogen in testes over time, while size and age*size do not (ANOVA).

Effect	SS	df	F	P-value
Size	8.95	1,19	1.35	0.26
Age	142.23	1,19	21.43	0.0002
Size*Age	4.97	1,19	0.75	0.40

Table 4. Age and size significantly predict % adult nitrogen in thorax over time, while age*size does not (ANOVA).

Effect	SS	df	F	P-value
Size	26.04	1,36	6.63	0.01
Age	26.82	1,36	6.83	0.01
Size*Age	9.7	1,36	0.81	0.12

Figure legends

Figure 1. Testes area increases over time, leveling off between 10-15 days of age. These results corroborate earlier results that show testes stop growing at about 11 days of age. Fitting a nonlinear model to testes growth shows that there are significant differences between small and large flies in terms of initial and final size, but not in growth rate.

Figure 2. Carbon turns over rapidly in testes. All initial carbon derives from larval sources, but carbon from adult sugar constitutes 52% of all testes C by day 5, and reaches a plateau above 60% between days 10 and 15. There are no differences between small and large flies for testes carbon.

Figure 3. Carbon turns over less rapidly in thorax than in testes. There were differences in thorax carbon turnover rates between small and large flies. All initial carbon derives from larval sources, but carbon from adult sugar constitutes 32% of all thorax C by day 5 for large flies, and 41% for small flies. Replacement of larval carbon plateaus at 41% of all thorax C for large flies, and 48% for small flies.

Figure 4. There is very little replacement of larval nitrogen with adult sources in testes tissue. Replacement is no different from zero until day 15, when nitrogen from yeast ingested at the adult stage reaches 7% of all testes nitrogen. There are no differences between small and large flies for testes nitrogen.

Figure 5. There is very little replacement of larval nitrogen from adult dietary sources in thorax tissue. Replacement is close to zero until day 15, when nitrogen from yeast ingested at the adult stage reaches 2.6 % of all testes nitrogen (small and large flies pooled).

Figure 6. The amount of nitrogen in thorax tissue was different between small and large flies over time. Note that the y axis in this figure reaches to only 6%; the figure is blown up in order to view differences between large and small flies.

Figure 7. Ratio of adult: larval grams of carbon invested in testes vs. thorax differs for small and large flies. Small flies invest relatively more grams of carbon from adult dietary resources into thorax, and less into testes, than do large flies.

Figure 1.

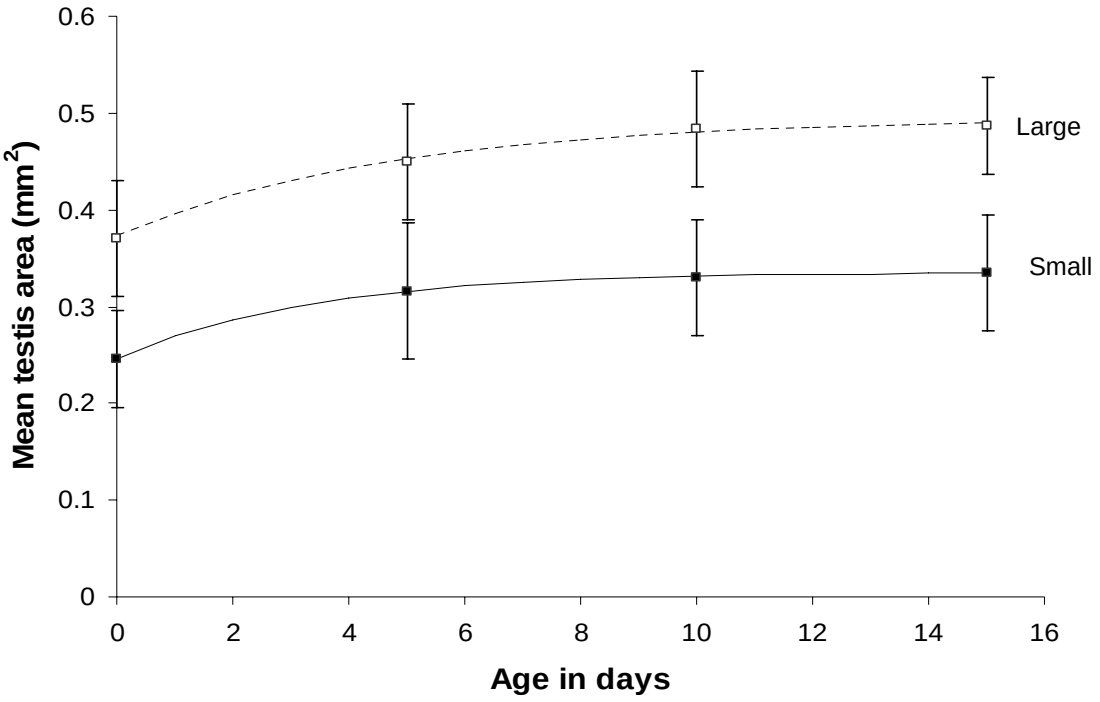

0.6
0.5
0.4
0.3
0.2
0.1
0
Mean testis area (mm2)
0
2
4
6
8
10
12
14
16
Age in days
Large
Small

Figure 2.

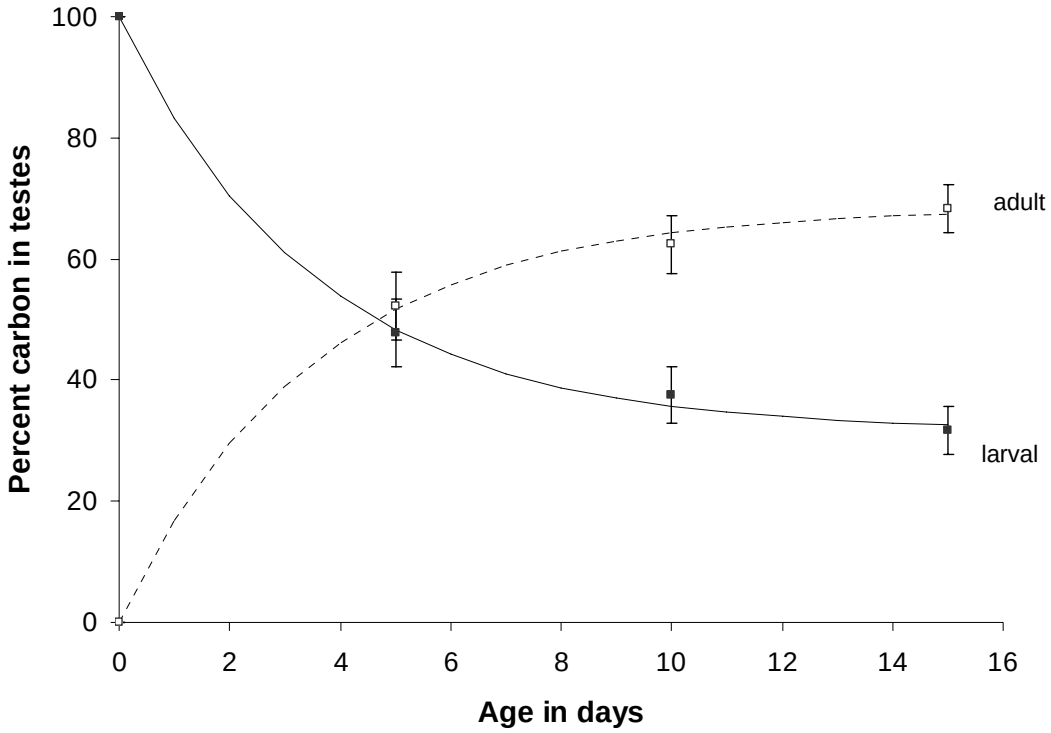

100
80
60
40
20
0
Percent carbon in testes
0
2
4
6
8
10
12
14
16
Age in days
adult
larval

Figure 3.

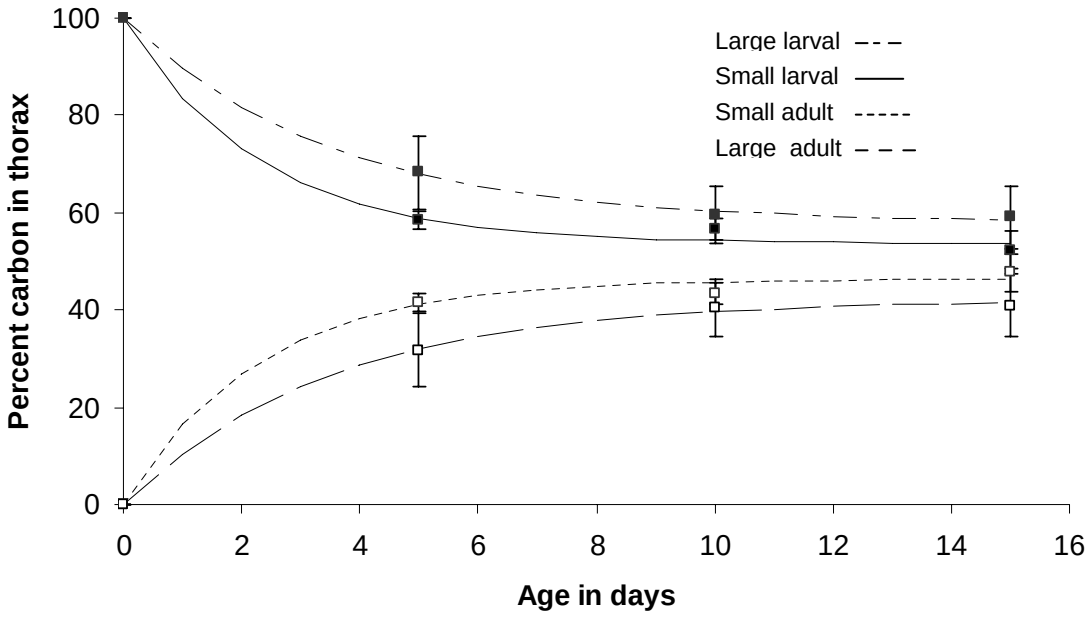

Large larval
Small larval
Small adult
Large adult
Percent carbon in thorax
Age in days
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16

Figure 4.

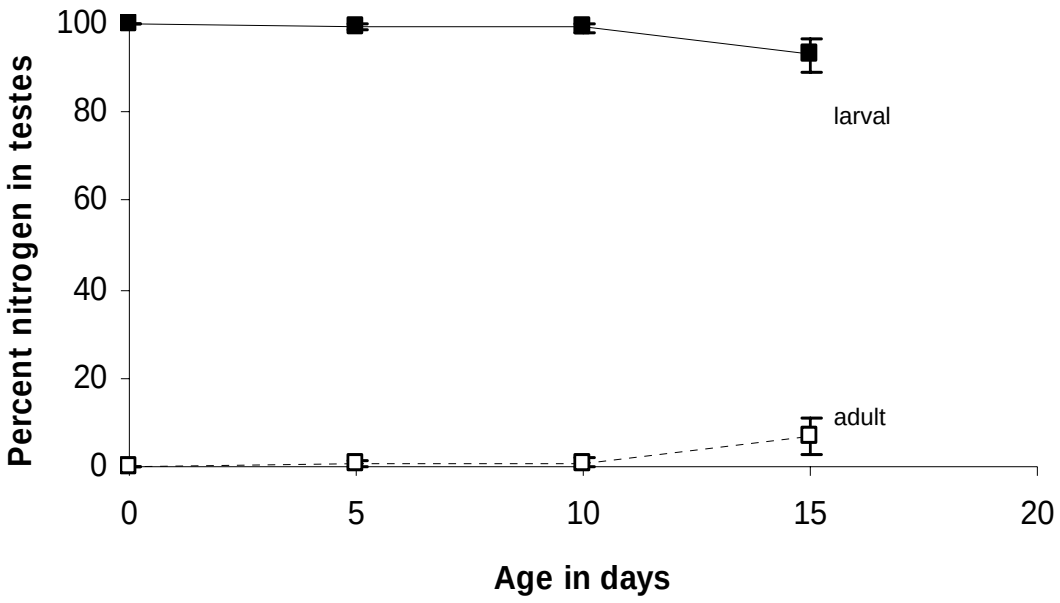

Percent nitrogen in testes
100
80
60
40
20
0
larval
adult
0
5
10
15
20
Age in days

Figure 5.

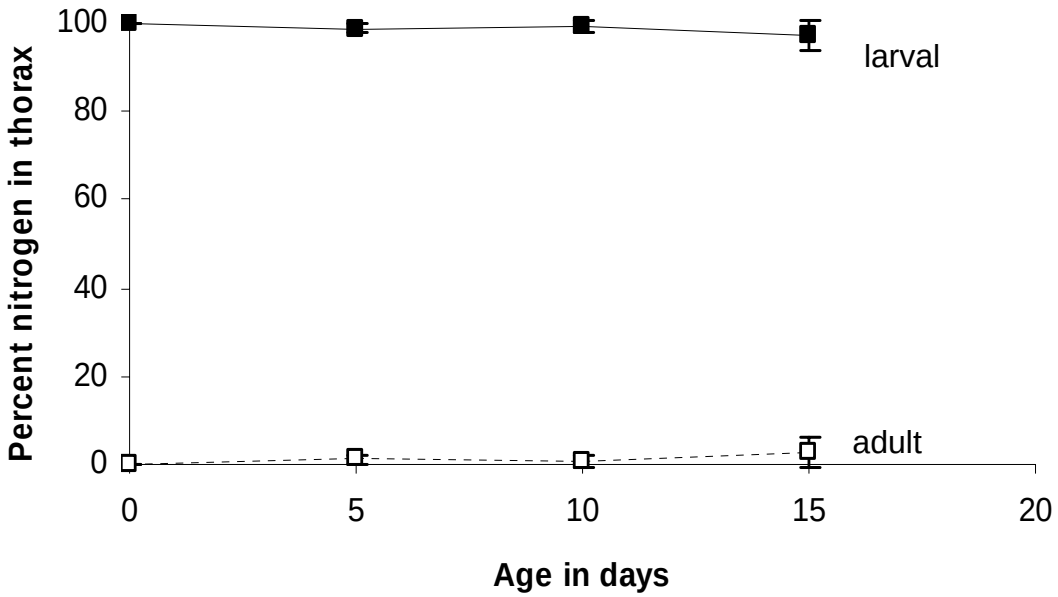

Percent nitrogen in thorax
100
80
60
40
20
0
0
5
10
15
20
Age in days
larval
adult

Figure 6.

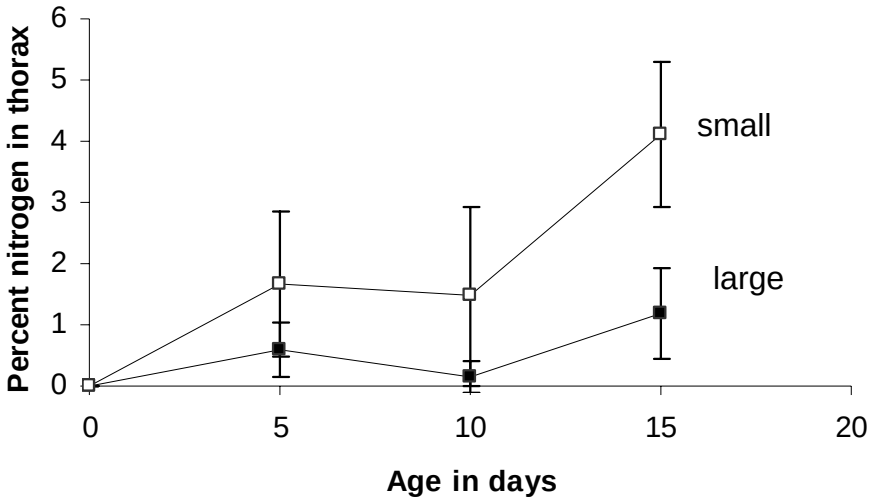
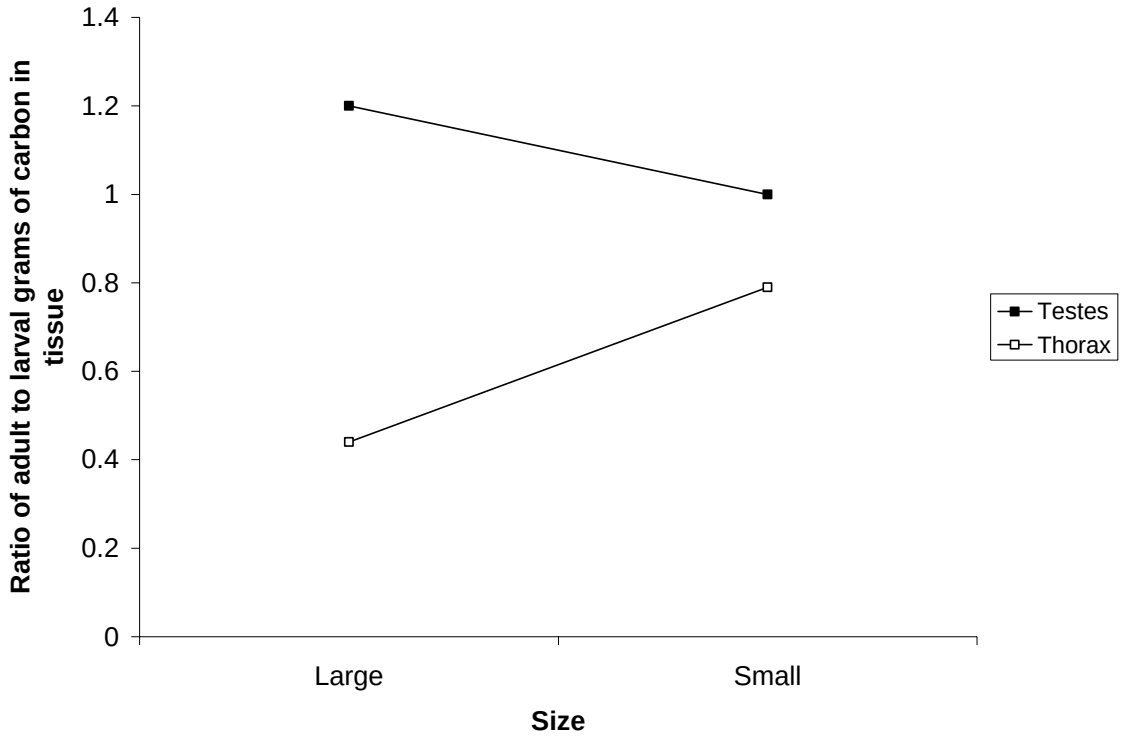

Percent nitrogen in thorax
6
5
4
3
2
1
0
0
5
10
15
20
Age in days
small
large

Figure 7.


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ratio of adult to larval grams of carbon in tissue
Large
Small
Size
Testes
Thorax

APPENDIX D

ROLE OF ADULT PROTEIN SOURCE IN MATING EFFORT AND TESTES SIZE

Role of adult protein source in mating effort and testes size

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Abstract

Sexual selection can act on males both before and after copulation, favoring different suites of reproductive traits. Although it would be beneficial for a male to invest maximally in traits that help him compete both before and after copulation, limitations in available resources might dictate that investment into one trait trades off against the other. I examined the relationship between mating success vs. investment into testes under conditions of unlimited access to hydrolyzed yeast (which contains amino acids and peptides necessary for protein synthesis) and conditions of yeast restriction in adult walnut flies, ***Rhagoletis juglandis.*** My results indicate that, when males have unlimited access to yeast, they increase mating effort at the expense of testes size. Conversely, when males have no access to yeast, they increase investment to testes but reduce mating effort. These results suggest that testes and mating effort may compete for proteinaceous resources within the organism.

Introduction

Successful copulation is a critical first step in male reproductive success. However, when females mate with multiple males, gaining a mate is no guarantee of fertilization. Competition between sperm within the postmating arena of the female reproductive tract can generate strong selection on traits that mediate fertilization success, such as testes size (e.g. Pitnick and Markow 1994).

In theory, it would benefit a male to invest maximally in traits that help him compete at both gaining copulations and competing during sperm competition. However, there are reasons to expect that investment in mating effort might come at the expense of investing in postmating traits. Life history theory predicts that when resources are limited, organisms must make allocation tradeoffs (Stearns 1992, Roff 1992). Sperm production can be costly (Dewsbury 1982, Pitnick 1996, Wedell et al. 2002), and if males spend copious amounts of time and energy on premating activities, such as courtship display (see Andersson 1994, Vehrencamp et al.1989 for costs of courtship), this may limit the amount of resources available to invest in traits that mediate sperm competition. The existence of a tradeoff between mating effort and postmating traits will depend on both the costs of those traits and the pool of resources available to an animal. If the traits are not costly, there should be no reason for a tradeoff: animals will invest maximally in both sets of traits. Similarly, under conditions of *ad libitum* resource availability, even costly activities may not result in an allocation tradeoff.

Previous studies have shown that adult nutrition (Mallard and Barnard 2004, Droney 1998), and in particular, access to protein (e.g. Kaspi et al. 2000), may play an

important role in determining different components of mating success, such as the ability to gain copulations or initiate courtship behavior. Thus, it appears that resource constraints can be important in mediating male mating success in some species. The effects of nutrient restriction on postmating traits are less studied, but allocation differences to testes in response to nutritional differences have been shown in at least one study (Droney 1998). While the effects of nutrient restriction on elements of mating effort and post mating success have been studied in isolation, there have been few attempts to examine how relative investment to each might interact.

Here, I investigate the relationship between testes size and mating effort in the walnut fly, *Rhagoletis juglandis,* under conditions of yeast restriction (hydrolyzed yeast contains amino acids and peptides, precursors for protein). I chose these traits because they are critically important in male reproductive success. Mating effort is important because males that fail to copulate have no hope of passing on their genes. Testes size increases in response to operational sex ratio (Carsten-Conner and Papaj, in review), suggesting that testes investment is biologically important in this species in order to mediate sperm competition. I chose to restrict access to yeast because protein is a critical resource among *Rhagoletis sp.*; females require protein to produce eggs (Prokopy and Papaj 2000). I hypothesized that the nutrients for building proteins found in yeast (amino acids and peptides) would be a critical resource for males, as well, limiting both mating effort and investment into testes when it is in short supply. I predicted that investment in mating effort and testes size would not tradeoff under control conditions of *ad libitum* yeast, but that they would when males were yeast deprived.

Methods

Study System

Rhagoletis juglandis engages in a resource defense mating system. Females lay large clutches of eggs (up to 30 eggs, Nufio et al. 2000) in walnut fruit (*Juglans major*) and males fight for the opportunity to guard oviposition punctures on fruit. Because females re-use oviposition punctures (Nufio et al. 2000), males that win contests and successfully guard punctures have increased mating opportunities (Papaj 1994). When females arrive at the fruit to oviposit, males approach females and rapidly vibrate their wings, producing a low frequency sound that may function as a courtship song (Alonso-Pimentel et al. 2000). These displays are often followed by copulation attempts by the males. Males invest considerable time in activities aimed at securing copulations. Both males and females remate frequently; thus, there is ample opportunity for sperm competition in this species. Testes size is relatively large in this species (about 1/3 the volume of the abdomen), suggesting that investment to testes may be costly.

Experiment 1

Flies were collected as pupae near Patagonia, AZ from a single population. Pupae were kept chilled at 4º C until ~ 4 weeks prior to the experiment, at which time they were warmed in a growth chamber (ca. 26º C) with 12 hours light:12 hours dark. Flies were allowed to emerge into clear plastic cages containing either 1) sugar, water, and hydrolyzed yeast (yeast plus treatment), or 2) sugar and water only (yeast minus

treatment). Within 48 hours of emergence, flies were transferred to clear plastic 473-ml cups with the same food and water regime as the cage into which they emerged. I established four yeast + cups and four yeast – cups, each containing 8 male flies. Flies were allowed to interact freely for 13 days after emergence, at which time they were killed by freezing. Previous studies indicate that testes grow for about 11 days past eclosion in this species (Carsten-Conner unpublished data); thus, flies would have reached maximal testes size by 13 days of age. Flies were dissected in glass depression slides with a drop of Ringer's solution (0.9g NaCl, 0.02g KCl, 0.4g dextrose, and 0.02g $CaCl_2$ in 100 ml deionized water), and their testes were extracted. I measured the area of digitized images of testes to obtain testes size. Both testes of each male were transferred to a compound glass slide and analogue video was recorded at 50x magnification under a dissecting scope. Analogue images of the testes were captured and digitized on an iMac, and ImageJ software (National Institutes of Health, version 1.32j) was used to estimate the total area encompassed by each testis. Mean testes area per male was used in the analysis. Mid-wing vein length was also recorded for each animal as a proxy for body size, as this measurement correlates well with body mass (H. Alonso-Pimentel, unpublished data).

I used a linear model to ask whether treatment and/or cup nested within treatment predicted testes area. I did not analyze the effects of body size within the model, as body size was equal between treatments (see results). Data were analyzed using JMP-IN statistical software (SAS Inc.; Cary, North Carolina, version 5.1.2).

Experiment 2

This experiment sought to test the effects of yeast limitation on mating effort. Flies were collected and reared as above. Again, males emerged into cages with access to either 1) water, sugar, and hydrolyzed yeast (yeast plus), or water and sugar only (yeast minus). Males were placed into clear plastic rearing cups within 48 hours of eclosion. Again, the yeast treatment in the rearing cups remained the same as that of the eclosion cage. For this experiment, 5 males were placed into each cup. All males in each cup were knocked out with CO_2 gas and assigned a color: yellow, blue, green, orange, or white. The color was applied as a small dot of tempera paint on the dorsal surface of the male, between the wings. For this experiment, I established a total of 10 cups, five protein plus and five protein minus.

On the third morning past eclosion, I added 5 females to each cup. I used females that emerged 8 days prior to the males in order to ensure that they would be sexually receptive (females take 7-9 days to mature in this species). Females were reared with free access to hydrolyzed yeast in order to ensure that any effects of yeast deprivation on mating were due to male behavior, rather than female behavior. The females were replaced halfway through the experiment with fresh, mature, yeast-fed females.

I began behavioral observations on Day 3 (72 hours post eclosion) at 10AM, one hour after females were added to the cups. Cups were observed 4 times a day for ½ hour periods between the hours of 10AM and 2PM. These hours represent peak times of behavioral activity for the walnut flies in the laboratory (L.Carsten-Conner, pers. obs.) All copulations in each cup were recorded. Observations were made on Days 3, 4, 6, and

8 past eclosion. Although males do not mature and engage in sexual activity until 3-5 days post eclosion, I chose to begin observations on Day 3 to evaluate whether or not onset of mating effort would be earlier in yeast plus males. Males were killed by freezing on day 13 and dissected as above in order to obtain testes size.

I used a linear model to ask whether treatment, day, and treatment*day predicted number of copulations. Data were log transformed (log (number of copulations +1)) to normalize the residuals. For testes size, we asked whether treatment and and/or cup nested within treatment predicted testes area (Again, body size was equal between treatments). There was no need for transformation of these data. Data were analyzed using JMP-IN statistical software (SAS Inc.; Cary, North Carolina, version 5.1.2).

Results

Experiment 1

Mean mid-wing vein measurement was 1.31 ± 0.01 mm. Body size was not different between the treatments (yeast plus mean mid-wing vein = 1.31 ± 0.01 mm; yeast minus mean mid-wing vein = 1.32 ± 0.01 mm; t-test, t= 0.35, df = 69, p = 0.73). Because there were no differences in body size, I used ANOVA to ask if there were differences in testes size as a function of yeast regime. Yeast fed males had significantly larger testes than yeast deprived males (ANOVA, $_{F1,63}$ = 48.2, p < 0.0001; Figure 1). There was no significant effect of rearing cup (p = 0.14).

Experiment 2

Color has been shown to create mating biases in some species (rev. in Ryan & Keddy-Hector 1992); thus, I tested whether there were mating differences according to color. There were no differences in copulation numbers among males of different colors (ANOVA, $F_{4,45} = 1.30$, $p = 0.28$).

Mean mid-wing vein measurement for Experiment 2 was 1.38 ± 0.01 mm. Body size was not different between the treatments (yeast plus mean mid-wing vein = 1.38 ± 0.02 mm; yeast minus mean mid-wing vein = 1.38 ± 0.02 mm; t-test, t= 0.54, df = 48, p = 0.59). In contrast to Experiment 1, there were no differences in testes size between yeast plus and yeast minus males (ANOVA, $F_{1,40} = 3.49$, $p = 0.07$, Figure 2). A comparison of testes size under conditions of yeast availability and yeast restriction shows that testes size for yeast plus males was smaller in Experiment 2 than in Experiment 1, and that testes size for yeast minus males was larger in Experiment 2 than in Experiment 1. Rearing cup was not significant ($p = 0.08$).

Mating effort generally increased through time, and the slope of the regression line differed between yeast plus and yeast minus males (Figure 3). There was a significant effect of treatment (ANOVA, $F_{1,192} = 5.66$, $p < 0.01$), Day (ANOVA, $F_{1,192} = 48.37$, $p < 0.0001$), and treatment * Day (ANOVA, $F_{1,192} = 8.54$, $p < 0.004$), in the model. Following up with an analysis of cumulative mating effort between yeast plus and yeast minus males revealed that yeast plus males engaged in significantly more copulations over the period of the study than did yeast minus males (t-test, t = -2.10, df = 198, p = 0.04). There were few copulations in either group on Day 3 and 4, as some males do not mature until Day 5. The differences in copulation number increased through time; thus,

males that are yeast deprived will likely engage in many fewer lifetime copulations than their yeast-fed counterparts.

Discussion

My results are consistent with the hypothesis that testes investment and mating effort trade off in this species, but the relationship between the traits is complex. When no females were present, yeast plus males grew testes that were about 1.3 fold larger than the testes of yeast minus males. This suggests that hydrolyzed yeast is an important factor in testes growth. It may be that amino acids and peptides (or another nutritional element of yeast) are actually limiting for testes growth in this species. Alternatively, it may be that nutrient deprived males choose to allocate their limited resources to other functions when mating opportunities are not available.

However, comparing testes size in the presence of females vs. the absence of females reveals two main trends: 1) testes size in yeast plus males decreased when females were present compared to when they were absent, and 2) testes size in yeast minus males increased when females were present compared to when they were absent. Concurrently, mating effort was higher in yeast plus males than in yeast minus males, increasing over time. These results are consistent with the idea that, when nutrients are limitless, males reduce allocation to testes in order to step up mating effort. For yeast minus males, however, it appears that testes allocation increases at the expense of mating effort. These results suggest that allocation of resources to testes and mating effort compete within the organism. These inferences are drawn by comparing results of

experiments run at separate times; however, the experimental conditions were fairly consistent. The experiments were run within weeks of each other in the same growth chamber, with identical temperature and humidity settings. Flies were drawn from the same source population, and body size was similar across treatments (mean in Experiment 1 = 1.32 ± 0.01 s.e., mean in Experiment 2 = 1.38 ± 0.01 s.e.).

The idea that two distinct activities, biosynthesis of tissue (testes growth) and energy metabolism (mating effort), compete for proteinaceous resources within the organism is somewhat problematic, as the currency used for each function generally differs. Nitrogen is used primarily for biosynthesis, while carbon is used for energy metabolism. However, because yeast contains both carbon and nitrogen, it is possible that the different components of yeast are routed to different functions within the organism, and that the tradeoffs indicated in this study actually reflect more complex nutrient routing within the flies. That is, various functions and activities, including mating effort, male-male contests, testes investment, and somatic maintenance, may receive higher or lower priority depending on nutrient availability.

It is interesting that the direction of the apparent tradeoff demonstrated in this study changes depending on nutrient availability. Because yeast plus males had unlimited access to yeast nutrients, we might expect that they would invest maximally to both testes and mating effort, and that no tradeoff would be evident. The fact that there is an apparent tradeoff even under these conditions suggests that one or the other activity bears very high physiological or energetic costs, which are paid in the currency of carbon and/or nitrogen. This is consistent with the results of Hunt et al. (2004), who found that

high quality crickets (protein fed) invested maximally in mating effort at the expense of a decreased lifespan.

For yeast minus males, the increased allocation to testes and decreased allocation to mating effort suggests that, if mating effort is very energetically expensive, then nutrient deprived males have no hope of competing at gaining mates. Thus, they may allocate more resources to testes in hope of winning at sperm competition among the mates they do secure. These results are consistent with those of Droney (1998), who found that among Hawaiian *Drosophila*, low quality males allocated relatively more to testes than did high quality males. In my study, testes size was no different between yeast plus and yeast minus males when females were present; thus, it appears that nutrient deprived males have little advantage over yeast plus males. However, I did not test these patterns under conditions of intermediate access to nutrients. In nature, it is unlikely that nutrient access is an "all or nothing" situation. Perhaps males with low, rather than no, access to particular nutrients will manage to grow larger testes than their nutrient-rich competitors.

Differences in allocation strategy among low vs. high quality males have been found in other species, as well. Among feral fowl, for instance, males least successful at gaining mates have more motile sperm (Pizzari et al. 2002). Among bluegill sunfish, large males court with greater success, but "sneaker" males produce more sperm per ejaculate (Fu et al. 2001), leading to greater postcopulatory success. These results, along with those of the present study, suggest that a general first-choice strategy of high-quality males may be investment into activities that lead to securing copulations, rather than

investment into traits that mediate sperm competition. Future studies should focus on the actual paternity rates accruing to high vs. low quality males in order to evaluate the relative importance of investment into mating effort vs. sperm competition for different species.

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Figure legends

Figure 1. Males reared without females and with free access to hydrolyzed yeast had significantly larger testes at 13 days of age than males reared without females and deprived of access to yeast.

Figure 2. Testes size was not different between males reared with females and free access to hydrolyzed yeast than males reared with females and deprived of access to yeast.

Figure 3. By Day 8, yeast plus males engaged in significantly more copulations than did yeast minus males.

Figure 1.

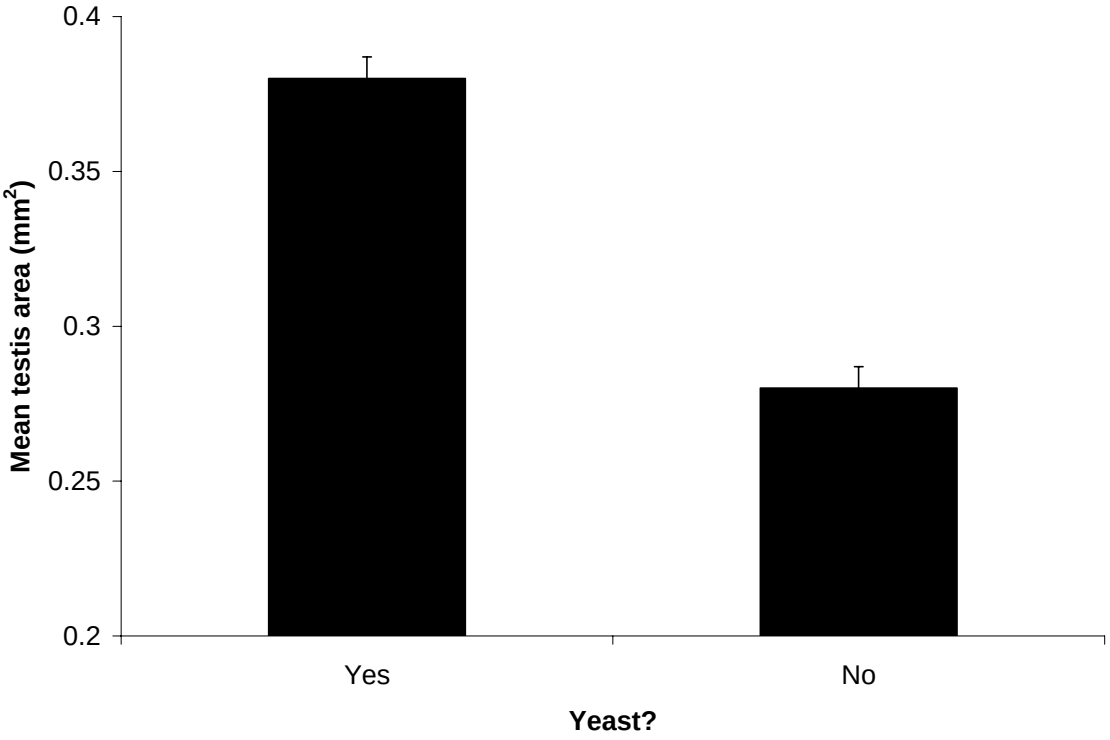


Figure 2.

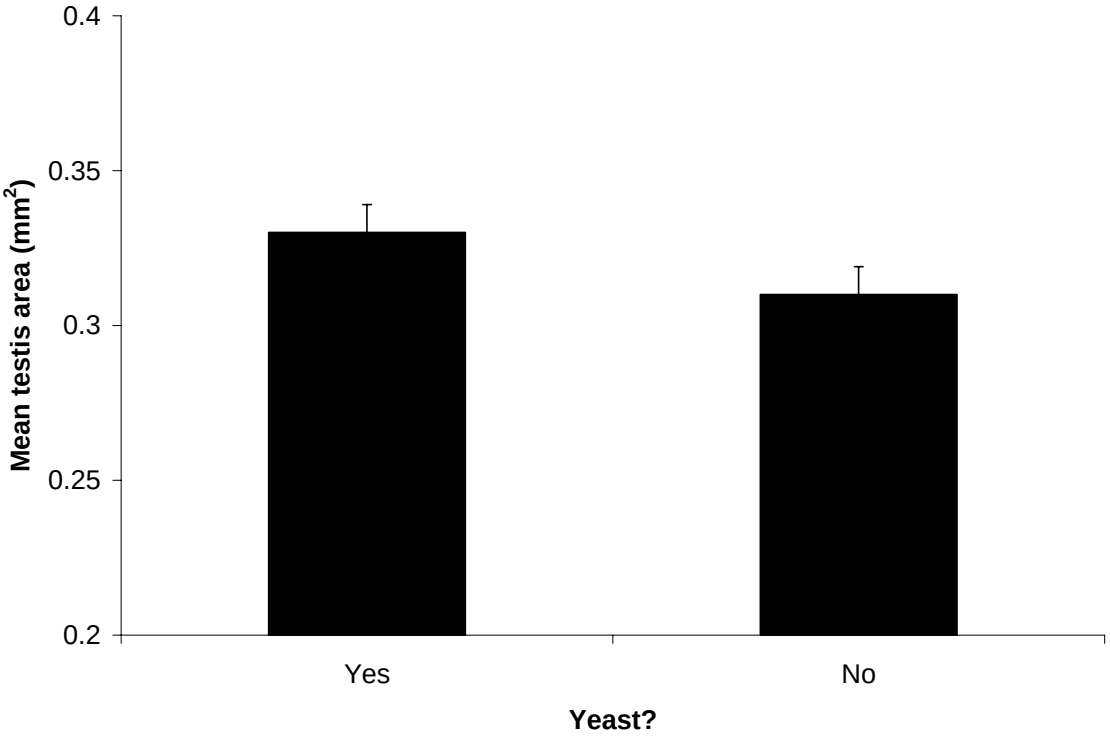
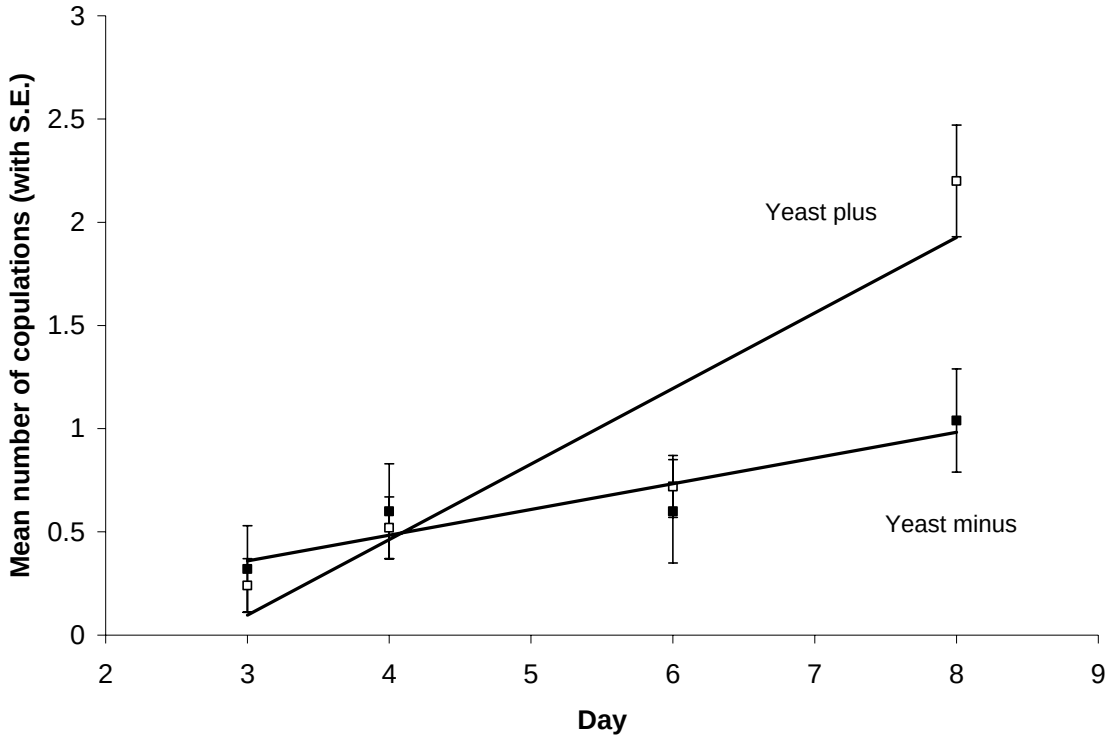


Figure 3.


Mean number of copulations (with S.E.)
3
2.5
2
1.5
1
0.5
0
2
3
4
5
6
7
8
9
Day
Yeast plus
Yeast minus

APPENDIX E

SUPPLEMENTAL DATA

SUPPLEMENTAL DATA

Methods

This experiment aimed to test whether large flies have increased mating success in comparison to small flies. I used flies collected from a single population in southern Arizona in 2003. Pupae were maintained at 4 ^{o}C prior to the study, and warmed to room temperature (ca. 29 ^{o}C) for approximately 5 weeks before eclosion. Upon eclosion, flies were maintained in plastic rearing cages provided with hydrolyzed yeast, sugar, and water. Male and female flies were separated from each other within 2 days of eclosion and maintained in separate cages. For the experiment, I set up 10 clear plastic 473-ml cups with a surrogate fruit (a 3.7-cm diameter yellow plastic sphere) suspended from the top of the cup. I placed one large male, one small male, and one female into each cup and observed mating behavior for 1 hour. I determined male size visually, using only flies at the large and small ends of the spectrum for this study. In every cup, the "large" male was distinctly larger than the "small" male, and errors in assignment during observations were negligible. I recorded which fly copulated first, total number of copulations for each fly in each cup, and the duration of the first copulation in each cup. The experiment was repeated 8 times. Data were analyzed using JMP statistical software.

Results

Overall, large flies were significantly more likely than small flies to achieve the first copulation in the cup ($\chi^2 = 13.6$, $p = 0.0002$, $df = 59$, $n = 60$; Fig. 1). Large flies gained

more copulations than did small flies, as well ($F_{1,59} = 5.6$, $p = 0.02$, $n = 60$; Fig. 2). In contrast, size did not predict duration ($\chi^2 = 0.32$, $p = 0.33$, $df = 59$).

Figure 1.

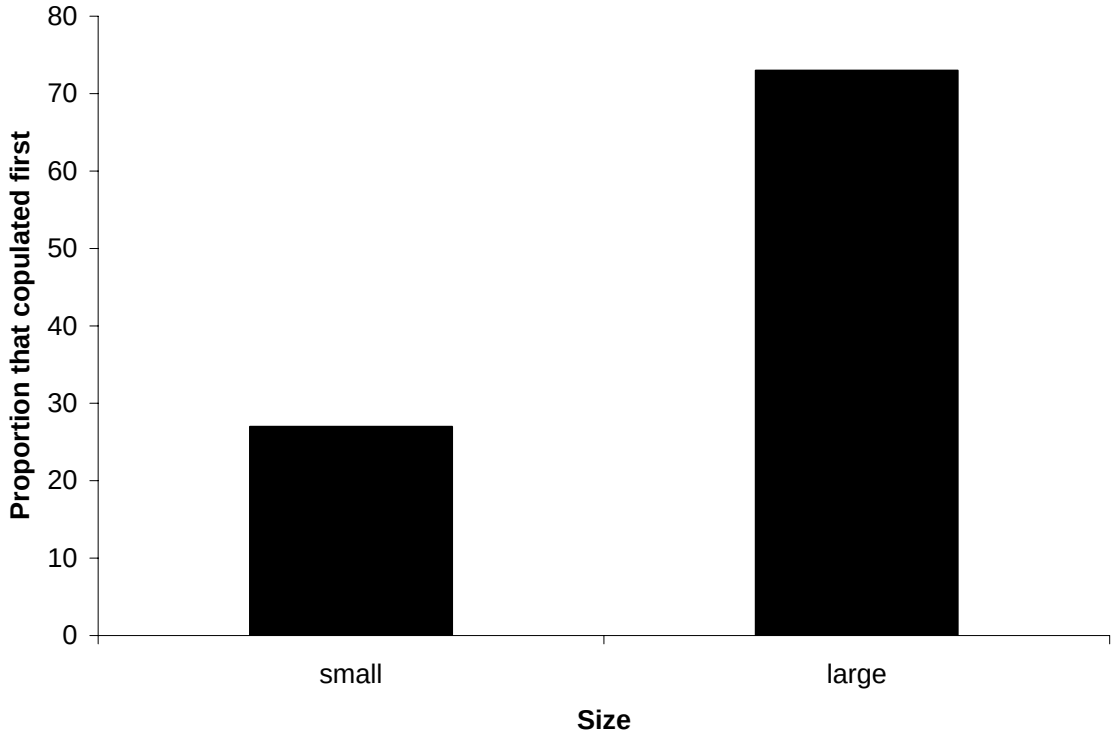
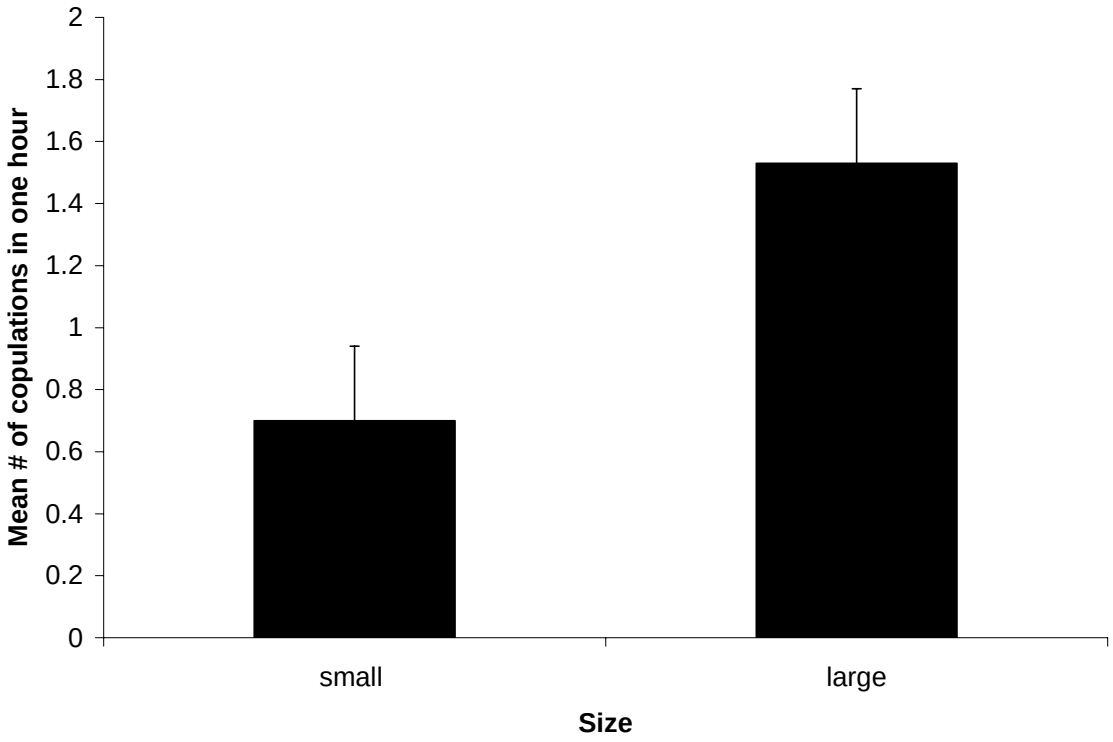


Figure 2.


Mean # of copulations in one hour
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
small
large
Size