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Resource allocation to testes in walnut flies and implications for reproductive strategy

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ABSTRACT

Testes size often predicts the winner 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 derive from the larval or adult diet. Distinguishing the source of nutrients allocated to testes can shed light on life history factors (such as maternal influences) that shape the evolution of male reproductive strategies. 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. We found significant incorporation of adult dietary carbon into testes, implying that walnut flies are income breeders for carbon (relying more on adult resources). In contrast, we found little evidence that walnut flies incorporate adult dietary nitrogen into testes development. We discuss the implications of these allocation decisions for life history evolution in this species.

1. 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 and Millar, 2004; Preston et al., 2003; Hosken et al., 2001; Stockley et al., 1997; Gage, 1994; Møller, 1991; Svärd and 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 development 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,

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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 (e.g., mean testis area among males in a 1:11 M:F sex ratio treatment was 0.54 mm, in contrast to a mean testis area of 0.60 mm among males in a 9:3 M:F sex ratio treatment; Carsten-Conner, Papaj, in preparation). 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 (testes continue to grow until ~11 days after eclosion in this species). However, investment constraints might arise through nutritional constraints imposed by diet quality. Walnut fly larvae solely ingest their natal host fruit. 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. Adult flies are thought to obtain carbohydrates primarily from fruit exudates and nitrogenous resources from ephemeral sources such as bird feces (Prokopy and Papaj, 2000). There are reasons to expect that small males will direct these 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 (in paired mating trials, about 28% of small flies gained mates compared to 74% of large flies; 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 postcopulatory 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 or traits. Here, we ask whether small flies can compensate for their size by allocating relatively more adult resources to testes than do large flies.

2. 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.

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

2.1. Experimental protocol

Flies were obtained from larval-infested fruit collected at sites in southern Arizona during the summer of 2005. Infested fruit were brought into the lab and held over sand until mature larvae emerged and pupated in the sand. Pupae were placed into cups and maintained at 4 °C for at least four months prior to the study, and warmed to room temperature (ca. 29 °C). Adult flies eclosed approximately 3 weeks after warming. Flies used in this study originated from multiple fruits from multiple trees. Flies originating from a given collection cup were distributed evenly across treatments. Within 48 h 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. Details on the growth of these labeled and unlabeled yeasts are given in O'Brien et al. (2008).

The flies were reared in an incubator kept at ca. 27 °C. One cup containing small flies and one cup containing large flies were collected and frozen at three different time intervals: 5, 10, 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 °C for over 24 h, 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 photographed testes to estimate testes area. We also photographed the wings of each fly in order to obtain an actual body size (post hoc to our visual estimates), as mid-wing vein length correlates well with body mass (H. Alonso-Pimentel, unpublished data). 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.

2.2. 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. The analytical setup consisted of a Costech ECS4010 Elemental Analyzer interfaced with a Finnigan Delta Plus XP isotope ratio mass spectrometer via the Conflo III interface (Thermo-Finnigan, Inc.,

Bremen). 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 ($\delta^{13}C = -15.8$, $\delta^{15}N = 7.0$) to assess analytical accuracy and precision; these gave values of $\delta^{13}C = -15.8 \pm 0.1‰$ (SD), and $\delta^{15}N = 7.0 \pm 0.2‰$ (SD).

2.3. Calculations

The isotope ratio of nitrogen or carbon in tissue 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 offset by 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:

$$\delta^{13}C_{tissue} = \delta^{13}C_{fruit}\left(\frac{\%C_{fruit}}{100\%}\right) + \delta^{13}C_{yeast}\left(\frac{\%C_{yeast}}{100\%}\right) + \delta^{13}C_{sugar}\left(\frac{\%C_{sugar}}{100\%}\right) + \Delta$$

$$100\% = \%C_{fruit} + \%C_{yeast} + \%C_{sugar}$$

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 and Rosenheim, 2005), and we solve for $\Delta = 0$, −1, and +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 (O'Brien et al., 2002):

$$\%C \text{ from yeast} = \frac{\delta^{13}C_{labeled\ tissue} - \delta^{13}C_{unlabeled\ tissue}}{\delta^{13}C_{labeled\ yeast} - \delta^{13}CC_{unlabeled\ yeast}} \times 100\%$$

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

$$\%N_{yeast} = \frac{\delta^{15}N_{labeled\ tissue} - \delta^{15}N_{unlabeled\ tissue}}{\delta^{15}N_{labeled\ yeast} - \delta^{15}N_{unlabeled\ yeast}} \times 100\%$$

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:

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

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 × %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. All statistical analyses were carried out using JMP-IN statistical software (SAS Inc.; Cary, NC, version 5.1.2). Results are reported with standard errors.

3. Results

3.1. Body size and testes growth

In this study, we visually categorized flies at eclosion as large vs. 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 vs. 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, $p < 0.0001$).

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 (0.24 ± 0.01 vs. 0.37 ± 0.01 mm^2, respectively) and final (0.34 ± 0.01 vs. 0.49 ± 0.01 mm^2, respectively) testes area, but not in growth rate (small $r = 0.30 \pm 0.13$, large $r = 0.21 \pm 0.06$).

3.2. 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

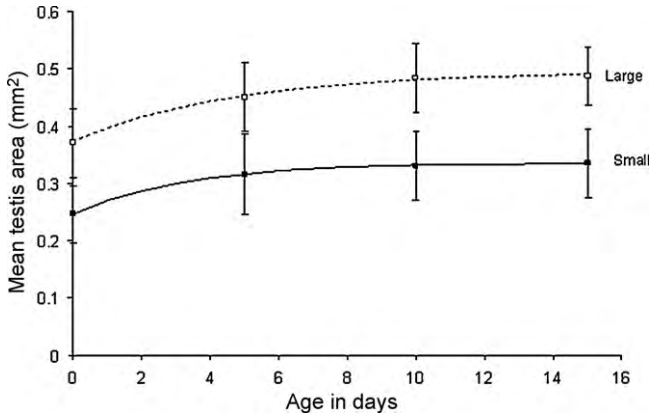

Fig. 1. Testes area increases over time, leveling off between 10 and 15 days of age. These results corroborate earlier results that show testes stop growing at about 11 days of age. Fitting a nonlinear model to testes growth shows that there are significant differences between small and large flies in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

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 days 0 and 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.

3.3. 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:

$$\%C \text{ from fruit} = \frac{(\delta^{13}C_{\text{tissue}} - \Delta) - \delta^{13}C_{\text{sugar}}}{\delta^{13}C_{\text{fruit}} - \delta^{13}C_{\text{sugar}}} \times 100\%,$$

where $\delta^{13}C_{\text{tissue}}$ is the isotopic ratio for carbon from males, $\delta^{13}C_{\text{sugar}}$ is the isotopic ratio of sugar carbon, $\delta^{13}C_{\text{fruit}}$ is the isotopic ratio for walnut fruit, and Δ gives the isotopic offset between diet and tissue, tested at three levels: −1‰, 0‰, and +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‰. If we assume a fractionation of ±1‰ (Spence and Rosenheim, 2005), 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

Table 2
Isotopic signatures of tissues in this study. Data are expressed in delta notation as $((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000$. 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.

Yeast	Nitrogen (δ^{15}N)		Carbon (δ^{13}C)	
	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

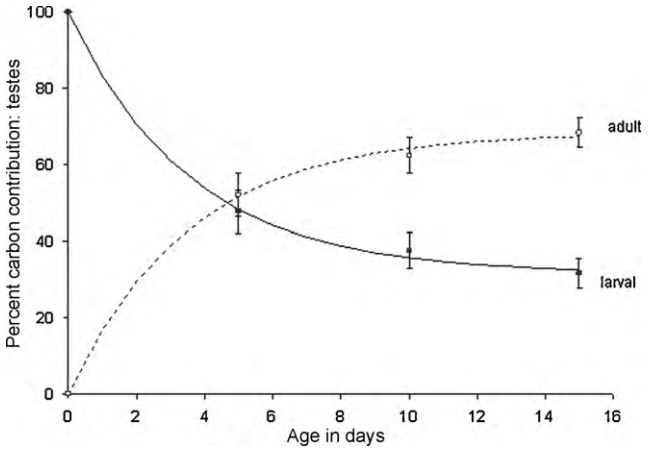

Fig. 2. Testes carbon turns over rapidly in testes with adult dietary sugar. All initial carbon derives from larval sources, but carbon from adult sugar constitutes 52% of all testes C by day 5, and reaches a plateau above 60% between days 10 and 15. There was no detectable incorporation of carbon from adult dietary yeast, and there were no differences between small and large flies in testes carbon turnover.

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 rate 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 to a lesser degree 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. Thus, while differing estimates of carbon isotope fractionation alter absolute values of carbon incorporation, the difference between large and small flies remains 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

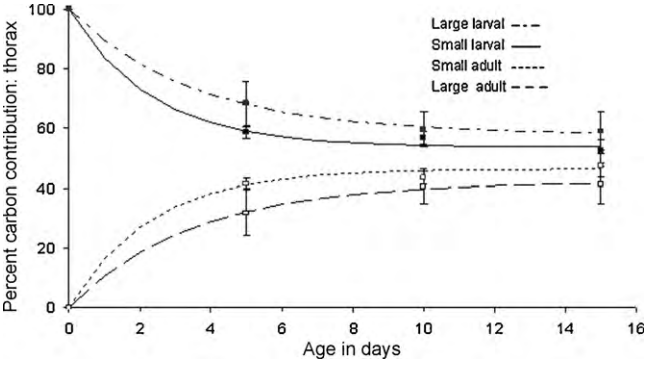

Fig. 3. Thorax carbon turns over with adult dietary sugar less rapidly than does testes carbon. There was no detectable incorporation of carbon from adult dietary yeast. There were differences in thorax carbon turnover rates between small and large flies. All initial carbon derives from larval sources, 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.

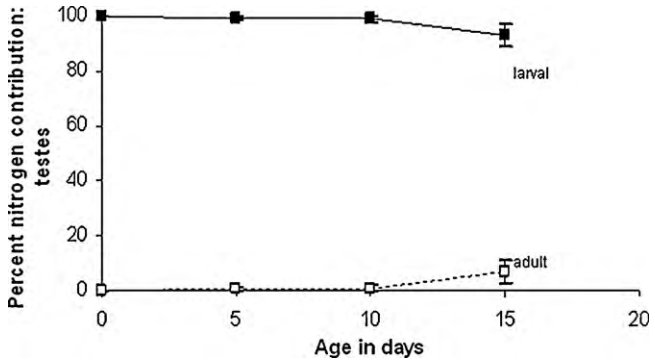

Fig. 4. There is very little replacement of larval nitrogen with adult sources in testes tissue. Replacement is no different from zero until day 15, when nitrogen from yeast ingested at the adult stage reaches 7% of all testes nitrogen. There are no differences between small and large flies for testes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

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.

3.4. Allocation of nitrogen to testes and thorax

In contrast to carbon, flies used little adult nitrogen 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. 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 and no interaction between age and size (Table 3).

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

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

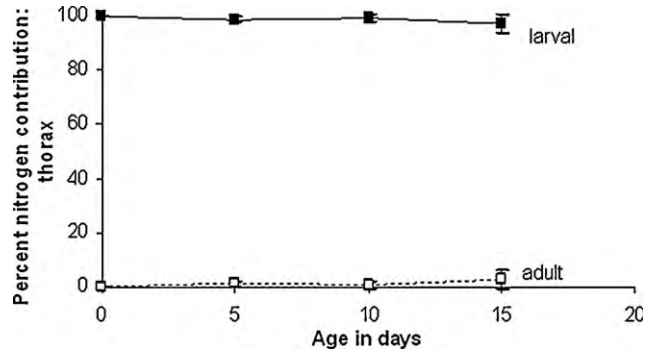

Fig. 5. There is very little replacement of larval nitrogen from adult dietary sources in thorax tissue. Replacement is close to zero until day 15, when nitrogen from yeast ingested at the adult stage reaches 2.6% of all testes nitrogen (small and large flies pooled).

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

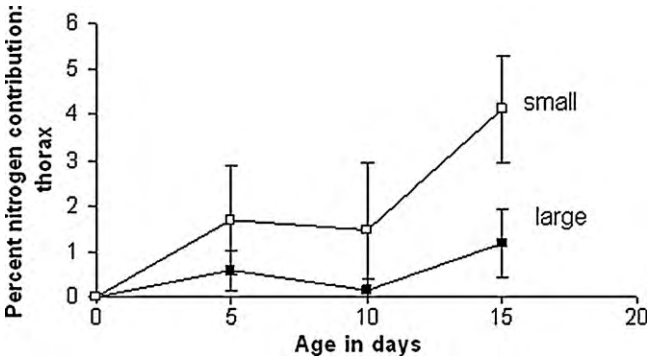

Fig. 6. The amount of nitrogen in thorax tissue was different between small and large flies over time. Note that the *y* axis in this figure reaches to only 6%; the figure is blown up in order to view differences between large and small flies.

flies averaged 2.16 ± 0.45% adult nitrogen in thorax, while large flies averaged 0.43 ± 0.43% (Fig. 6).

3.5. Relative allocation to each tissue

We compared relative allocation of resources to testes vs. thorax for large vs. small flies by comparing the ratio of adult to larval investment (in grams C or N) in each structure for each size class. Small flies allocate more total adult carbon to thorax, and less total adult carbon to testes than do large flies (Fig. 7). There were no differences in adult: larval nitrogen (g) allocated to testes vs. thorax for small vs. large flies.

4. Discussion

4.1. Overall allocation strategy

Walnut flies appear to be income breeders with respect to carbon. Testes carbon turned over rapidly, reaching a final

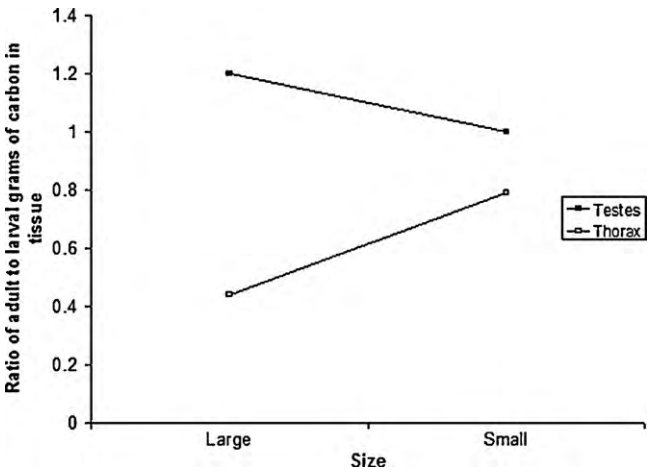

Fig. 7. Ratio of adult: larval grams of carbon invested in testes vs. thorax differs for small and large flies. Small flies invest relatively more grams of carbon from adult dietary resources into thorax, and less into testes, than do large flies.

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. This finding supports previous results (e.g., Droney, 1998, Carsten-Conner unpublished data), that dietary quality in the adult stage affects male testes development among dipterans and points to dietary carbon as a key resource. Because dipterans primarily use sugars to fuel energy metabolism and flight (Candy, 1989), expensive contest behaviors in males are likely to require income carbon for energy metabolism (Marden and Waage, 1990; Parker and Thompson, 1980). Our finding that male testes development also uses income carbon is consistent with a reliance on adult dietary sugar sources (such as fruit exudates, the suspected adult carbon source for *Rhagoletis*) for male reproductive behavior and development overall, and underscores how life history strategy can be linked to nutrient availability across an animal's life stages.

The relatively low carbon to nitrogen ratio in testes found in this study (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. The low incorporation of nitrogen from adult dietary sources into testes and thorax indicates that under the conditions of the present study, male walnut flies are capital, rather than income, breeders with respect to nitrogen. This finding begs the question of whether low reliance on dietary nitrogen sources is typical of this species in nature, or whether the finding might be an artifact of the nitrogen source provided in this study (whole yeast). In a previous study, *Drosophila melanogaster* readily incorporated nutrients from this experimental yeast into tissues (O'Brien et al., 2008); however, *Drosophila* has a very different nutritional ecology than *Rhagoletis jugulandis* and may be more poised to take advantage of yeast and other microorganisms as food sources. In a follow-up experiment we found that some females achieved full reproductive development on our experimental yeast (which requires a dietary source of protein, Prokopy and Papaj, 2000) while others did not (unpublished data). This suggests that *Rhagoletis* flies are capable of ingesting the experimental yeast, but the males nonetheless incorporated very little into their testes.

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). These resources are likely to be ephemeral and thus perhaps only opportunistically used, in contrast to walnut fruit exudates (suspected carbon source), which are widely available. Thus, the strategy of high reliance on income carbon, but low reliance on income nitrogen, may reflect true conditions in nature. More information about the relative amounts of carbohydrate-rich vs. protein-rich food sources ingested in nature would help reveal how these elements are functionally used. For instance, the flies may be using a strategy of constrained intake with nutrient interconversion, or they may be using foraging strategies that lead to a balanced intake of nutritionally complementary foods (see Raubenheimer et al., 2009 for a discussion of the geometric model of nutritional ecology).

4.2. Implications for the evolution of male reproductive strategies

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. Such a strategy might increase chances of success in the postcopulatory arena. Contrary to this prediction, there were no differences in carbon or nitrogen turnover (larval to adult) in testes tissue between small and large flies. We also found that small flies allocate relatively more adult carbon to thorax, and less to testes than do large flies. It may be that nitrogen limitation, particularly in flies with poorer larval nutrition, limits the extent of testes development in the adult stage and hence of carbon use in testes. Alternatively, if small flies engage in more energy-demanding behaviors (e.g., flight, contests, etc.), they may require more resource investment in flight muscle maintenance and repair (O'Brien et al., 2008).

Taken together, our results suggest that ultimately, despite their ability to use income resources towards testes growth, testes size in walnut flies is largely determined by larval environment. To the extent that increased testes size translates into a postcopulatory advantage as it does in other species (e.g. Stockley and Purvis, 1993; Pitnick and Markow, 1994; Schärer et al., 2004), larval nutrition is likely to be 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 testes, and small flies do not appear to be capable of compensating for this size-imposed disadvantage. 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 an oviposition decision that results in decreased offspring size may also be detrimental to offspring sperm competitive ability; which may increase selection pressure for production of high-quality (e.g. large) offspring.

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