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Biological stoichiometry of growth in *Drosophila melanogaster*

Thomas Watts[a], H. Arthur Woods[b], Sarah Hargand[a], James J. Elser[c], Therese A. Markow[a,*]

[a]*Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721-0088, USA*
[b]*Section of Integrative Biology, University of Texas at Austin, Austin, TX 78712, USA*
[c]*Department of Biology, Arizona State University, Tempe, AZ 85287, USA*

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Abstract

We examined the relationship between growth rate, C:N:P stoichiometry, and nucleic acid content in *Drosophila melanogaster*. The "Growth Rate Hypothesis" predicts that N and P contents per unit body mass will be high during ontogenetic stages characterized by rapid growth, reflecting the large requirement for P-rich ribosomal RNA during these periods. The ratio of RNA:DNA also is predicted to change with changes in growth rate.

Growth is rapid in early *D. melanogaster* larvae, slowing considerably just prior to pupation. As predicted, a positive relationship was found between growth rate and N and P content, but not C. Thus, body C:P and N:P ratios declined with increasing growth rate. The relationship between RNA content and growth rate also was positive. Additionally, the fraction of total body P contributed by ribosomal RNA increased with increasing growth rate.

Keywords: Nitrogen; Phosphorus; RNA; Growth rate

1. Introduction

Recent studies have proposed that inter- and intraspecific differences in growth rate are associated with differences in elemental composition (C:N:P stoichiometry; Elser et al., 1996, 2000b; Hessen and Lyche, 1991; Sterner, 1995). The association arises because growth requires increased allocation to structures, primarily ribosomes, which are disproportionately rich in P. Furthermore, those cells with relatively high ribosome content also may be N-rich due to contributions of ribosomal proteins, RNAs and protein synthetic products. Thus, there should be a generally higher nutrient content in rapidly growing organisms or tissues and the effects should be especially strong for phosphorus because rRNA can dominate cellular P pools (Sterner and Elser, 2002).

This proposed relationship, termed "the Growth Rate Hypothesis," provides a framework for translating organismal traits such as growth, cellular allocations, and biochemical composition in N and P content to ecological studies of the storage and flows of multiple chemical elements (Melillo et al., 2003). For example, species or ontogenetic stages having high N and/or P content are more likely to experience food quality limitation when food nutrient content declines (Sterner and Elser, 2002). In addition, because growth rates are central to life history theory, the hypothesis provides a means of linking organismal elemental composition to evolutionary questions. For example, what adaptations underlie life history differences in organisms associated with high and low food quality as measured by N and P content? How is the evolution of life history variation in response to food quality constrained by an organism's elemental composition? What is the genetic architecture of evolutionary responses to food quality differences?

For example, in insect herbivores, speciation is often associated with shifts to novel host plants (Berenbaum, 2002). Because terrestrial plant species vary considerably in their elemental composition (Sterner and Elser, 2002), selection should favor the evolution of adaptations that enable herbivores to utilize new plant resources of differing

*Corresponding author. Tel.: +1 520 626 3323; fax: +1 520 626 3522.
E-mail address: tmarkow@arl.arizona.edu (T.A. Markow).

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nutritional quality (Fagan et al., 2002; Jaenike and Markow, 2003). Understanding the genetic underpinnings of these adaptations is critical to our ability to deal with insects of economic importance, with loss of biodiversity, as well as to discover basic principles of ecological speciation.

Support for a general association of rapid growth rate with elevated RNA content, and perhaps P contents, can be inferred from studies of crustacean zooplankton (Dagg and Littlepage, 1972; Båmstedt and Skjoldal, 1980; McKee and Knowles, 1987; Main et al., 1997; Gorokhova and Kyle, 2002; Andersen and Hessen, 1991; Hessen and Lyche, 1991; Elser et al., 2000a; Gorokhova et al., 2002, Vrede et al., 1998). Elements of the GRH also are supported by the basic biochemistry of insect growth (Niemierko, 1952; Lang, et al., 1965; Church and Robertson, 1966a, b; Medrano and Gall, 1976), the physiology of nutrition and feeding (Dadd, 1985, Simpson and Simpson, 1990), and correlations between growth rate and RNA content (Devi et al., 1963). In general, most insect larvae exhibit higher mass-specific rates of growth early in development (Slansky and Scriber, 1985). RNA contents also generally are higher earlier in development (Lang et al., 1965; Price, 1965; Church and Robertston, 1966b). None of these studies, however, directly test the GRH. First, body mass data from early in development generally lack sufficient detail to permit accurate calculation of early mass-specific growth rates. Second, previous studies have not measure both biochemical and elemental contents that are necessary for a quantitative test of the GRH.

Here we test directly the GRH in *Drosophila melanogaster* by asking whether ontogenetic changes in growth rate are predictably associated with changes in larval elemental and nucleic acid contents. Because growth rates of *Drosophila* larvae, measured as the mass-specific rate of change, are highest during the earliest stages of growth and subsequently decline (Church and Robertston, 1966b), we expect these early stages to have greater relative P and RNA contents than in later, more slowly growing stages.

2. Materials and methods

2.1. Culture of Drosophila

Flies used in this experiment were drawn from a mass culture established from an isofemale line collected in Panama in 1999 by TAM. To initiate each experiment, parental flies were harvested on the day they eclosed, separated by sex, and kept in yeasted banana vials for 3–5 days. Approximately 20 females and 20 males were mated for 24 h in shell vials containing 0.5% agar. Five of these vials of mated flies ($n = 200$ flies) then were pooled and then equally distributed to each of four half-pint milk bottles (50 flies per bottle). These bottles were inverted over 60-mm plastic Petri dishes containing 25 g of yeasted standard banana food. After ovipositing for 3 h, adults were removed. Larval densities were well below competitive levels.

2.2. Growth rate determination

Two runs were performed for each growth rate experiment. In *D. melanogaster*, first instar larvae hatch at approximately 22–24 h after oviposition. Starting at 36 h post-oviposition, larvae were harvested every 12 h until the majority of remaining larvae had pupated. Therefore, larvae at the first sampling time were approximately 12 h old (± 1.5 h). At each sampling time point, larvae were plucked from the medium with forceps, allowed to "swim" for 10 min in deionized water to remove any adhering food or substrata, and then were placed in the appropriate vessel for further analysis. Six samples per time point, each containing multiple larvae, were dried at 50 °C for 48 h and weighed. In order to have enough material for the various analyses, earlier time points necessarily contained more individuals than later ones. For each time point, the six dried samples were used to calculate growth rate as

$$\mu(\mathrm{d}^{-1}) = \ln[\mathrm{mass}_{x+24}/\mathrm{mass}_x]/\mathrm{d}t,$$

where mass_x is body mass on a given time x, mass_{x+24} is mass at $x+24$ h, and $\mathrm{d}t$ is the time interval between the body mass measurements (1 day). Note that though samples were collected every 12 h, growth rate was calculated by comparing samples that were collected 24 h apart.

2.3. Elemental analysis

Material from three of the six dried samples was subsequently used to determine N and C contents. Dried and weighed samples contained in tin capsules (Alpha Resources, Stevensville, MI) were processed for carbon and nitrogen content by a Finnegan MAT Delta Plus© continuous flow mass spectrophotometer fitted with a Costech Element Analyzer© in the Department of Geosciences at the University of Arizona. The remaining three dried and weighed samples were digested with persulfate, and phosphorus concentration was determined colorimetrically using the ascorbic acid method (APHA, 1992).

2.4. Nucleic acid analysis

For nucleic acid determinations, groups of three larvae per time period were snap frozen in groups of three in 1.5-ml microcentrifuge tubes and stored at –80 °C until used. Nucleic acid concentrations were assayed using a Ribogreen reagent assay developed for use with small insects (Kyle et al., 2003). Triplicate samples were assayed for each time point.

2.5. Statistical analyses

All elemental and biochemical contents were expressed as a percentage of total body dry mass. Curve fitting analysis and statistical curve comparisons were performed using GraphPad Prism v. 4.03 for Windows (GraphPad Software, San Diego, CA www.graphpad.com). Most appropriate curves for regressions were identified using an F test (extra sum-of-squares).

3. Results

3.1. Growth rates

Mass specific growth rates of *D. melanogaster* larvae were calculated for the two experimental runs (Fig. 1). While the same pattern, high initial growth rates that decreased with time (1: $F_{1,19} = 96.45$, $p < 0.0001$; 2: $F_{1,19} = 223.75$, $p < 0.0001$) was observed in both experimental trials, the relationship was stronger in the second run. Nonetheless, the difference between the two runs was statistically insignificant (slope: $F_{1,38} = 1.24$. $p = 0.27$; intercept: $F_{1,39} = 2.08$, $p = 0.16$). Pupation occurred between 108 and 120 h post-oviposition. Growth rates slowed considerably 24 h prior to pupation, when larvae stopped feeding and moved around the Petri dishes searching for pupation sites. Though both growth rate curves appear to tail off at the end, an F test (extra sum-of-squares) indicates that a linear regression is the most appropriate (1: $F_{1,18} = 3.68$, $p = 0.071$; 2: $F_{1,18} = 0.57$, $p = 0.46$).

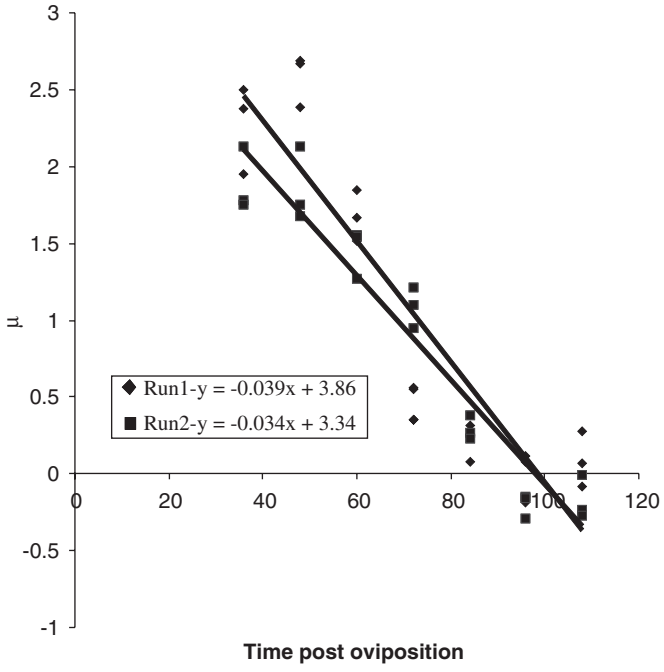

Fig. 1. Growth rate (μ) of larval *D. melanogaster* from 36 h post-oviposition to pupation. Time is in hours. Growth rate is defined as $\ln[\text{mass}_{x+24}/\text{mass}_x]/dt$ where mass is dry mass and dt is the duration (1 day) of the observation interval.

3.2. Elemental composition

To test for the predicted association between growth rate and contents of C, N and P, various measures of elemental composition were regressed against mass-specific growth rate (Fig. 2a). For each regression, the most appropriate regression model as determined by F test (extra sum-of-squares) is illustrated in the figure and the corresponding equation is shown below each graph. Both experimental runs showed essentially the same pattern: rapid growth was associated with higher percentages of N (1: $F_{2,18} = 7.98$, $p = 0.003$; 2: $F_{2,16} = 17.17$, $p < 0.0001$) and P (1: $F_{1,16} = 15.14$, $p = 0.001$; 2: $F_{1,18} = 73.16$, $p < 0.0001$), while the opposite relationship was observed for C (1: $F_{2,18} = 35.17$ $p < 0.0001$; 2: $F_{2,16} = 121.57$, $p < 0.0001$). The two runs were statistically identical for C ($F_{3,34} = 2.33$, $p = 0.09$) and P (Slope: $F_{1,34} = 0.28$, $p = 0.60$; intercept: $F_{1,35} = 0.09$, $p = 0.77$), but not for N ($F_{3,34} = 5.72$, $p = 0.003$). Coupled with the growth rate analyses, these associations indicate that fast growing, relatively young larvae are somewhat low in C relative to slower growing late stage larvae, while N and P show the opposite relationship. The C:P (1: $F_{1,16} = 14.12$, $p = 0.0017$; 2: $F_{1,17} = 71.82$, $p < 0.0001$), C:N (1: $F_{2,18} = 34.53$, $p < 0.0001$; 2: $F_{2,16} = 33.38$, $p < 0.0001$) and N:P (1: $F_{1,16} = 8.72$, $p = 0.009$; 2: $F_{1,17} = 15.85$, $p = 0.001$) ratios (Fig. 2b) were also inversely associated with growth. The two runs were statistically identical in the case of C:P (Slope: $F_{1,33} = 0.17$, $p = 0.69$; intercept: $F_{1,34} = 0.42$, $p = 0.52$) and N:P (Slope: $F_{1,33} = 0.12$, $p = 0.73$; intercept: $F_{1,34} = 0.54$, $p = 0.47$), but not in the case of C:N ($F_{3,34} = 7.00$, $p = 0.0009$). The significant decline in N:P with growth rate indicates that proportional increases in P content with increasing growth rate were larger than proportional increases in N content.

3.3. Nucleic acid content

RNA content was approximately 10% of dry body weight at 36 h, peaked at about 15% at 48 h, and then declined steadily as the larvae approached pupation. DNA concentrations reached a maximum (~1% of dry body mass) at 36 h and decreased steadily over the larval life span. This difference in RNA and DNA content is reflected by the different scales in Fig. 2c. Both RNA content (1: $F_{1,15} = 12.89$, $p = 0.003$; 2: $F_{1,19} = 104.66$, $p < 0.0001$) and DNA content (1: $F_{2,18} = 19.76$, $p < 0.0001$; 2: $F_{2,18} = 204.92$, $p < 0.0001$) increased with growth rate. Comparisons between runs indicate a similar slope ($F_{1,33} = 4.06$, $p = 0.052$), but dissimilar intercept ($F_{1,34} = 4.13$, $p = 0.049$) for RNA, but similar curves For DNA ($F_{3,36} = 0.40$, $p = 0.75$). Although both RNA and DNA content were positively correlated with growth rate in both runs, RNA:DNA ratios (Fig. 2c) showed no association with growth rate in the first replication ($F_{1,15} = 0.009$, $p = 0.925$) but a significant correlation for RNA:DNA ($F_{2,18} = 27.74$, $p < 0.0001$) in the second.

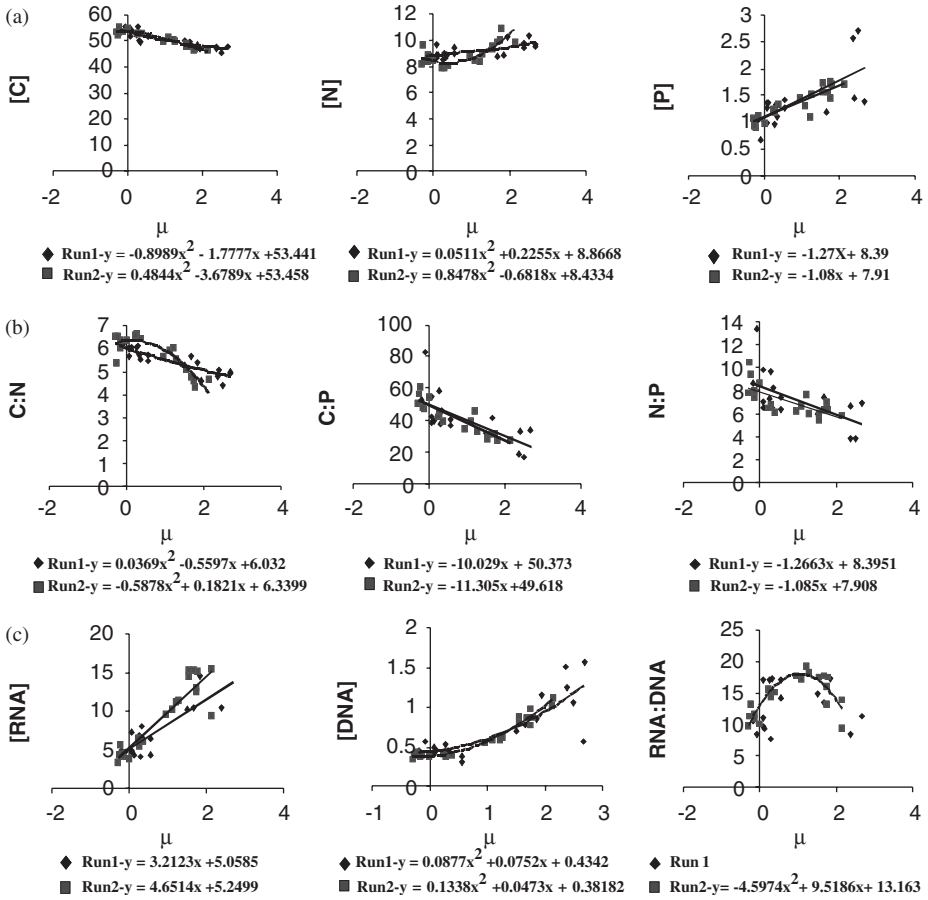

Fig. 2. Growth rate of larval *D. melanogaster* regressed against various elemental (P, N, and C) and biochemical contents. All contents are expressed as percent of dry mass.

The relationship between total body P and total P sequestered in RNA, and the relationship between growth rate and P sequestered in RNA, are shown in Fig. 3a and 3b respectively. With respect to total body P and P in RNA, the relationship was significant in both experimental runs (1:$F_{1,14} = 5.93$, $p = 0.029$; 2: $F_{1,18} = 75.97$, $p < 0.0001$). The diagonal line represents the line at which all body P would be sequestered in RNA. A comparison of the two runs indicates that the curves are statistically distinct (Slope: $F_{1,32} = 11.1$, $p = 0.002$; intercept = NA). Over the lifespan of *D. melanogaster* larvae, RNA represents a significant (30–85%) fraction of the total P pool. The association between growth rate and % of total P contributed by RNA was not significant in the first run ($F_{1,14} = 2.48$, $p = 0.137$), but positive in the second ($F_{1,18} = 30.43$, $p < 0.0001$).

4. Discussion

The GRH (Elser et al., 1996, 2000b) predicts that fast-growing organisms, or stages in an organism's ontogeny, should exhibit a distinct chemical signature, characterized by high RNA and P content. We observed a very large ontogenetic decrease in growth rate in *D. melanogaster*. Across development, N, P, RNA, and DNA contents all were positively associated with growth rate and, like growth rate, declined during ontogeny. These trends support the GRH in this ontogenetic context.

While there is some possibility of auto-correlation artifacts in these analyses due to the shared parameter of dry weight involved in the content and growth rate determinations, such artifacts did not influence our findings. First, autocorrelation between growth rate measures and measures of nutrient or RNA contents should produce negative correlations because the shared parameter (body dry weight) is in the numerator of the growth rate calculation but in the denominator of the expression for nutrient or biochemical content. However, we observed strong *positive* correlations of growth rate with key content parameters (e.g. %P, %RNA). Second, autocorrelation artifacts were not influencing our comparisons of body P content and P-content due to RNA, as plots of body RNA and body P for age-matched samples, uncorrected for dry mass, also showed strong positive correlations. Thus, we are in a position to interpret these data relative to how they reflect on the validity of the GRH in this ontogenetic setting.

While most of the measured variables changed as a function of growth rate, not all are equally likely to be associated to the process of growth. Of the nucleic acids,

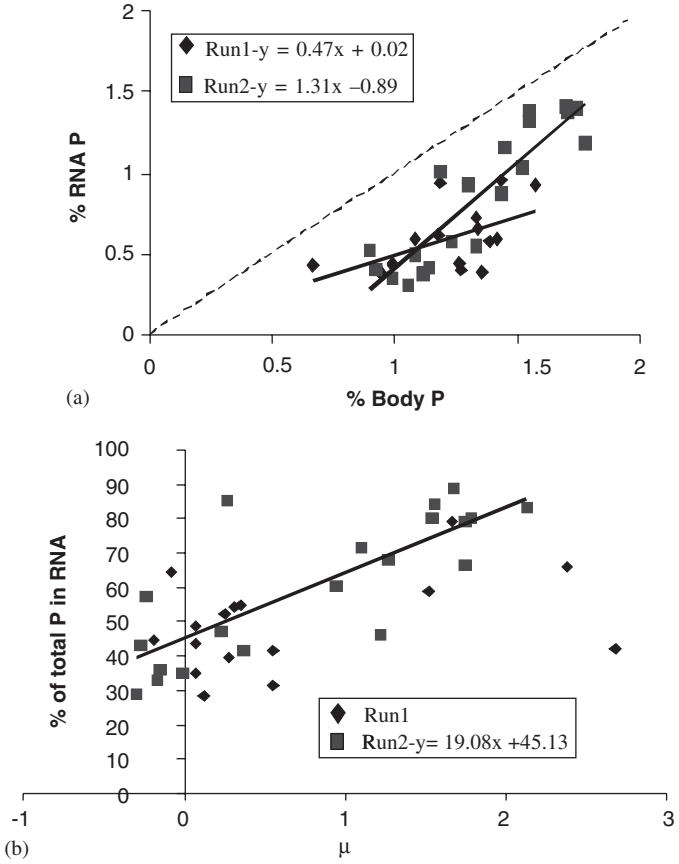

Fig. 3. (a) Total body P-content regressed against the content of RNA-P. Contents are expressed as percent of dry mass. The dashed line indicates a 1:1 relationship of RNA-P vs. total P; (b) growth rate regressed against the percent of total body P that is contributed by RNA.

RNA content is more likely than DNA content to be closely related to growth because of the demands for increased protein synthesis during accelerated growth. RNA content was 5- to 8-fold higher than DNA content and also varied more than DNA content. Because about 85% of cellular RNA is ribosomal (Watson et al., 1988), the changes in RNA content that we observed likely reflect shifts in rRNA associated with changing cellular demands for biosynthesis. DNA content, however, also was associated with growth rate, being relatively high during early stages of rapid growth.

In *Drosophila*, larval growth is thought to occur primarily by increases in cell size rather than cell number (Robertson, 1976). Furthermore, in *Drosophila* as in other Diptera, cells in certain tissues are highly polyploid, inflating the DNA content beyond what is expected in normal diploid somatic cells (King, 1968). The ontogenetic drop in DNA content, however, indicates that the total amount of larval DNA does not increase as rapidly as the mass of other larval components. The slight increase in DNA content at pupation probably reflects cell division associated with growth of imaginal discs (Robertson, 1976).

All of the elements measured (C, N, and P) were significantly correlated with growth. However, variation in P content was much greater (>2-fold) than variation in N (~30%) or C (~15%) content. Thus, P appears to be more directly associated with growth processes. This makes sense, as P and RNA are coupled currencies: nucleic acids contain significant amounts of P in the sugar–phosphate backbone, and can represent large fractions of the total P in the organism (Elser et al., 1996). Furthermore, because RNA content was much higher than DNA content (5–15% and <1%, respectively), variation in RNA probably was a more significant contributor to observed fluctuations in P content during ontogeny. Indeed, direct calculations from our measurements indicate that 35–80% of total larval P is contributed by RNA. In addition, the fraction of P in RNA increased with increasing growth rate (Fig. 3). Although N content also correlated with growth rate (Fig. 2), it varied over a smaller range. This result is consistent with previous work on *D. melanogaster* by Church and Robertston (1966b), who showed that protein content of larvae varied little over ontogeny. In our study, larval C content was negatively correlated with growth rate. This likely reflects a relatively modest allocational shift from biochemicals with relatively high C contents to those with lower C content. For example, proteins and nucleic acids differ in C content (53% C vs. 32% C, respectively; (Sterner and Elser, 2002) and thus the decrease in C content may reflect a decrease in the protein/nucleic acid ratio, or a decrease in the relative abundance of lipids (~70% C) at high growth rate. However, while a modest increase in lipid content as ontogeny proceeds may explain the modest decrease in N over time, it cannot account for the dramatic decreases in P, RNA and DNA seen here.

Insect gut contents are a potential confounding factor in a study of this type. Estimates of gut capacity of 2nd and 3rd instar *D. melanogaster* have been made on a number of lines using radio-labeled yeast (Miranda and Eggleston, 1988). These authors estimated a mean gut capacity of 2nd instar larvae (53 h post oviposition) of 6.8 μg (range 2.0–10.1 μg), and for 3rd instar larvae (77 h post oviposition) of 31.4 μg (range 19.3–43.4 μg). Using the dry weight/wet weight ratio of the food used in this study and the dry weights of the larvae used here, we can estimate that gut contents contribute to 0.6–3.3% of dry weight of 2nd instar larvae and 0.4–2.2% for 3rd instar larvae. These negligible numbers are unlikely to skew our data to a significant degree. Late third instar larvae, which stop feeding for 12–24 h prior to pupation, do not exhibit significant changes in our measured biochemical indices when compared to their immediate predecessors.

In summary, chemical composition correlates with growth rate in *D. melanogaster*. High growth rates are associated with high RNA, high P and low C:P and N:P ratios. These observations provide a means of linking ontogenetic shifts in biochemistry to important ecological and evolutionary issues. For instance, because early stage larvae are more N- and P-rich than later stages, they may

require higher dietary availability of N and P (Sterner and Elser, 2002). This could result in ontogenetic bottlenecks, as has been observed in copepod development (Villar-Argaiz and Sterner, 2002) or adaptations in oviposition site preferences or life history characters. For example *D. melanogaster* breeds in necrotic fruit. These resources are high in N and P relative to the resources used by cactophilic *Drosophila* (Markow et al., 1999). Female *D. melanogaster* prefer to oviposit in freshly wounded fruit, avoiding more necrotic pieces already colonized by other, competing arthropod species (Markow, 1988). In the four cactophilic *Drosophila* species endemic to the Sonoran desert, however, less than half of the available resource patches are occupied by either adult flies or larvae (Breitmeyer and Markow, 1998). Reduced patch occupancy does not appear to be associated with other factors, such as competitors or predators (Castrezana and Markow, 2001), but could reflect a lack of attractiveness, based upon inter-patch variability in nutritional quality, to ovipositing females. Furthermore, in species specializing on nutrient-poor hosts, such as the cactophilic *Drosophila* (Markow et al., 1999), first instar larvae may themselves show P-positive chemotaxis or compensatory feeding (Simpson and Simpson, 1990). Another mechanism by which early larval demands for P can be met is through egg provisioning. Support for the occurrence of maternal provisioning of P comes from a study of seven species of *Drosophila* (Markow et al., 1999), in which females of all species were found to have higher P content and lower N:P than conspecific males. *Drosophila* ovaries contain highly polyploid nurse cells that synthesize large quantities of P-rich RNA that ends up in developing oocytes (King, 1968). Additionally, in two species of *Drosophila,* females have solved their need for P for oogenesis by obtaining it directly from the seminal fluid of their mates (Markow et al., 2001).

Evolution of interspecific variation in life history traits, such as development time, body size, reproductive maturity and fecundity may also be predicted to be driven by food quality. Species of the genus *Drosophila* differ dramatically in the elemental composition of their hosts (Markow et al., 1999; Jaenike and Markow, 2003), their development times (Markow and O'Grady, unpublished), and in their reproductive biology. No studies have yet addressed whether variation in these life history traits is related to stoichiometric differences among host types. For example, larval growth rates are predicted to be slower in species adapted to nutrient-poor hosts, such as cacti (Markow et al., 1999) compared to the more rapid development time expected in mycophagous *Drosophila* that breed in N- and P- rich mushrooms (Jaenike and Markow, 2003), although the relationships between growth rate and biochemical composition should be similar. Additionally, the genetic architecture underlying differences in growth rate may also be associated with host-type. Specifically, according to arguments presented by Elser et al. (2000b), rDNA copy number and intergenic spacer length should both be greater in species with comparatively rapid growth. Using data for *D. melanogaster* as a baseline, and controlling for phylogenetic relationships, we are now able to begin testing these predictions in other, ecologically diverse *Drosophila* species.

Acknowledgments

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