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Ontogenetic coupling of growth rate with RNA and P contents in five species of *Drosophila*

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Summary

1. We studied the associations among growth rate, RNA content and P content at ~12-h intervals during the larval stage in five species of Drosophilids that specialize on host foods that differ substantially in P content.

2. Consistent with expectations based on the 'growth rate hypothesis' (GRH), within each species there were significant positive correlations between growth rates and RNA and P contents and in each species variation in P content was largely determined by differences in RNA content. However, there was a significant difference among species in how these three parameters were associated with each other, primarily due to differences in the intercept of the relationships rather than in their slopes.

3. Consistent with the GRH, we also observed positive associations among the average growth rates, RNA contents and P contents of the five species. Furthermore, these differences were broadly consistent with differences in the P content of their host resources: for example, *Drosophila falleni*, a species that specializes on P-rich mushrooms, had the highest growth rates and P and RNA contents while *D. pachea* and *D. mettleri*, species that specialize in low-P exudates from necrotic cacti and trees, had the lowest growth rates and P and RNA contents.

4. While data for additional species are needed, our findings provide further evidence consistent with the GRH and highlight a potential role of P limitation in shaping growth rate evolution in the *Drosophilids*.

Key-words: Biological stoichiometry

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Introduction

Understanding the ecological forces that drive and constrain the evolution of organismal growth and development rate is an important aspect of evolutionary ecology (Arendt 1997), as growth rate impinges on a variety of important life-history traits (e.g. age and size at first reproduction) and ecological features (e.g. predation risk, ability to exploit ephemeral resources) of a species. While the advantages of having a high potential for rapid growth and development seem obvious, species do differ considerably in their maximum rates of growth (Arendt 1997; Elser *et al*. 2000b) and thus it seems likely that there are important ecological factors that restrain growth capacity. However, the basis of these limits is not obvious. Recent work in the area of biological stoichiometry (Elser *et al*. 2000b; Sterner & Elser 2002) has suggested that one potential basis for a limitation on the evolution of rapid growth rate arises from the biochemical and cellular machinery that underlie organismal growth. That is, in the growth rate hypothesis (GRH, hereafter), differences in growth rate are associated with increased P requirements because organisms must disproportionately increase their allocation to P-rich ribosomal RNA to meet the protein synthesis demands of rapid growth rate (Elser *et al*. 1996; Sterner & Elser 2002). Thus, rapidly growing organisms must build unusually P-rich biomass and, all else being equal, this makes them more likely to suffer P-limitation because of an insufficient P supply in the external environment or diet (Sterner & Elser 2002).

A variety of lines of evidence have begun to emerge that support the GRH in different contexts (Elser *et al*. 2003). For example, significant associations between growth rate, RNA content and/or P content have been shown for crustacean zooplankton in interspecific or interclonal comparisons (Main *et al*. 1997; Elser *et al*. 2000a; Gorokhova *et al*. 2002; Vrede *et al*.

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1998; Weider *et al*. 2005; but see DeMott & Pape 2005) and in physiological studies (Acharya *et al*. 2004; Vrede *et al*. 2002). Some studies, again primarily involving crustacean zooplankton, have also begun to assess whether an animal's body P content affects its response to food with low P content. For example, a compilation of existing food quality studies suggests a positive relationship between body P content and the degree of reduction in growth rate in response high dietary C:P ratio in various zooplankton species (Sterner & Elser 2002), while a field study showed that fast-growing, P-rich arctic members of the *D. pulex* species complex showed stronger reductions in growth rate in response to decreased food P content than their low-P temperate counterparts (Elser *et al*. 2000a).

As indicated by the studies just mentioned, much of the work evaluating the GRH and the importance of dietary P in evolutionary ecology has been pursued in crustacean zooplankton. Thus, the relevance of these ideas for other groups of organisms, such as insects, is not apparent. However, it is clear from previous work that growth rate and RNA allocation are related in diverse biota (Sutcliffe 1970; Elser *et al*. 2000b, 2003). Recent observational (Schade *et al*. 2003) and experimental (Perkins *et al*. 2004) data do suggest that dietary P can be an important limiting factor for terrestrial insects as for zooplankton and thus it seems likely that P-limitation may also shape growth rate evolution in insects. This possibility would be bolstered by data showing that biomass P content was strongly coupled to biomass RNA allocation and growth rate in insects and especially if those relationships might also be shown to be related to an insect's trophic ecology.

Species of the genus *Drosophila* represent an attractive target for such tests, as they exhibit great diversity with respect to their dietary niche. Some are dietary generalists, while others may be associated with only one particular host resource. Although all *Drosophila* species are saprophytic, the decaying material they utilize can be very different in chemical composition, especially in P content (Markow *et al*. 1999; Jaenike & Markow 2003). For example, necrotic cacti and tree exudates are very low in P, while mushrooms are quite high (Jaenike & Markow 2003). In addition to their ecological diversity, the phylogenetic relationships of hundreds of *Drosophila* species are well established and genetic tools previously available only in *D. melanogaster* are rapidly being developed for other Drosophilids. We already have established that body P content of adults of different *Drosophila* species exhibits remarkable and significant variation, and that this variation is positively correlated with the P content of the host resources of the particular species (Markow *et al*. 1999; Jaenike & Markow 2003). What is not known, however, is if and how these species differences in dietary specialization are related to growth and RNA and P contents during larval development. If stoichiometric constraints are important as a selective agent in nature, then we would predict that Drosophilids that specialize on P-rich foods should have higher maximum growth rates during ontogenetic development and thus should exhibit high body RNA and P contents as larvae. In contrast, species that specialize on poor-quality, low-P foods should be characterized by lower growth rates, RNA content and P content in larval stages.

Having previously established the baseline relationships between growth rate, P and nucleic acid content in larval *D. melanogaster* (Watts *et al*. 2006), in this study we examine the associations among growth, RNA and P in four additional species that vary widely in resource use. *D. hydei* is a cosmopolitan species that is associated with decaying fruits, *D. pachea* and *D. mettleri* are both cactophilic species endemic to the Sonoran Desert of North America but are unrelated to each other (Markow *et al*. 1999), while the last species, *D. falleni*, is a mycophagous generalist occurring in forests of north-eastern United States (Jaenike 1978). These species therefore include two (*D. pachea* and *D. mettleri*) with an obligate association with a low-P host (necrotic cacti), one (*D. falleni*) with an obligate association with a high-P host (mushrooms), and a third (*D. hydei*) with a generalized diet having intermediate P content relative to these other two types of species (as in the case of *D. melanogaster*). Our study compares the growth and stoichiometry trajectories of these species on laboratory diets customized for each taxon. These diets are formulated to provide a different set of necessary phagostimulants and dietary microconstituents (e.g. specific sterols) so that each taxon develops at maximal rates. Thus, the diets differ not only in these biochemical constituents but also in overall nutrient (P) content, preventing a direct comparison of animal performance of the different species under identical dietary conditions. Given previous cautions regarding possible effects of diet on phenotypic traits (Conover & Schultz 1995), we call this to the attention of the reader as our study cannot distinguish possible effects of lab-rearing diet from taxon-specific traits because it is not currently possible to raise these species on uniform diets.

Materials and methods

STUDY SPECIES

Drosophila melanogaster were from a mass culture established from an isofemale line collected in Panama in 1999 by TAM. *Drosophila hydei* were from a mass culture established from an isofemale line collected in the Santa Rita Mountains, Arizona, USA, in 1999 by TAM. *Drosophila falleni* were collected in northern New York state, USA, in 2003 by J. Jaenike (University of Rochester). *Drosophila pachea* were collected near La Paz, Baja California Sur, Mexico, in 1998 by TAM. Finally, *Drosophila mettleri* were collected in the Superstition Mountains, Arizona, USA, in 1997 by S. Castrezana (University of Arizona).

REARING AND SAMPLING

All species were reared in the lab under low-density conditions in 300-ml glass bottles in a species-appropriate medium. *D. melanogaster* and *D. hydei* were reared in a standard banana/*Opuntia* medium (flyfood.arl.arizona.edu/opuntia.php3). *D. pachea* were reared on the same medium supplemented with a small amount (approximately 5 ml 60 g^{-1}) of the exudate of autoclaved Senita cactus (*Lophocereus schottii*). *D. mettleri* were reared on a potato/saguaro medium (flyfood.arl.arizona.edu/saguaro.php3), and *D. falleni* were reared on banana/*Opuntia* food supplemented with pieces of skinned, raw mushroom (*Agaricus bisporus*).

For all species used in this study, adults were collected on the day of eclosion, separated by sex and kept in yeasted banana/*Opuntia* vials until sexually mature. On the day of oviposition, males and females were allowed to mate and females were then allowed to oviposit on an appropriate medium in 100-mm plastic Petri dishes for 3–5 h. Adults were then removed. Oviposition medium was banana/*Opuntia* for *D. melanogaster* and *D. hydei*, banana/*Opuntia* supplemented with Senita exudates as above for *D. pachea*, potato/saguaro medium for *D. mettleri* and raw, skinned mushroom pieces for *D. falleni*. In all cases, the amount of larval food supplied was in gross excess of larval needs for the duration of the experiment.

GROWTH RATE DETERMINATIONS

Two runs were performed for each species. In the five species studied, first instar larvae hatch at approximately 22–24 h after oviposition. Starting at 36 h after oviposition, larvae were harvested every 12 h until the majority of remaining larvae had pupated. For species that took greater than 200 h to reach pupation, the sampling interval was increased to 24 h after the 200-h mark. Thus, the duration of the experiment differed for the five species (from 108 h for *D. falleni* and *D. melanogaster* to 324 h for *D. pachea*). Therefore, larvae at the first sampling time were approximately 12 h old (±1·5 h). At each sampling period, 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 placed in the appropriate vessel for further analysis. Three samples, each containing multiple larvae, were taken for determination of body mass at each time point. These were dried at 50 °C for 48 h and weighed and analysed for P content (see below). In order to have enough material for analyses, earlier time points necessarily contained more individuals than later ones. Three sets of three additional rinsed larvae were snap-frozen in liquid nitrogen for later analysis of RNA and DNA contents (see below). Weights obtained from the dried samples were then used to calculate growth rate for each interval as:

$$\mu\ (\text{day}^{-1}) = \ln(m_{x+1}/m_x)/T,$$

where m_x is body mass at a given time x, m_{x+1} is mass at the following time, and T is the interval between the body mass measurements (in days). Note that although larvae were harvested at 12-h intervals, growth rate calculations were made on larval samples collected 24 h apart in order to express growth rate in a standard manner.

PHOSPHORUS AND NUCLEIC ACID ANALYSES

Material from the three dried samples for each sampling point was digested with persulphate and phosphorus concentration was determined colorimetrically using the ascorbic acid method (APHA 1992). Nucleic acid contents of snap-frozen larvae (held at –80 °C until analysis) were determined using a Ribogreen reagent assay developed for use with small insects (Kyle *et al.* 2003). All chemical measures were then expressed as percentage of dry body mass (P content, RNA content). To estimate the percentage of total body P contributed by P in RNA (%P in RNA), the mean RNA content for a given species at a given sampling time was converted into P units using a conversion factor of 0·086 (RNA is ~8·6% P by mass; Sterner & Elser 2002) and then compared with that species' mean P content for that sampling point.

STATISTICAL ANALYSES

The raw data for growth rate, RNA content, P content and %P in RNA were first screened for statistical outliers (Mahlenhobis distance greater than 4). Data analyses were performed for the data set as a whole and also for data screened to included data for which all species had comparable body mass (<250 mg, as determined by the prepupation size of the smallest species, *D. falleni*). Both sets of data were subjected to analysis of variance (ANOVA) to examine the main effects of experimental run, time during ontogeny and species, along with their interactions.

To examine if species differed in how efficiently RNA and P allocations were coupled to growth rate, we calculated the mean growth rate, RNA content, P content and %P in RNA for each time interval for each species, keeping the data for the two independent runs separate. Using these averaged data we then performed analysis of covariance (ANCOVA) with μ as a dependent variable and species and RNA content, P content or %P in RNA as independent variables and including a species–μ interaction term. If the species–μ interaction was significant, this was interpreted as indicating that the species differed in the slope of their μ vs RNA, P, or %P in RNA relationships. If the interaction term was non-significant for a given dependent variable, we then repeated the ANCOVA using species as an independent variable and μ as a covariate. In this case, a statistically significant effect of 'species' would indicate

that the species differ in how growth is coupled to RNA, P or %P in RNA, in which the difference arises from the intercepts of the growth relationships. A similar analysis was performed to evaluate if the species differed in their P content *vs* RNA content relationships. As in the overall ANOVAS, all of these analyses were performed both for the overall data set and for data confined to animals <250 mg.

Interspecific patterns were also evaluated by taking overall means for each variable (μ, %RNA,%P, %P in RNA) for each of the five species over each species' entire ontogenetic development period, pooling the two experimental runs. We then examined the statistical significance of correlations among the averaged parameters considered across the five species.

All statistical analyses were performed using the JMP™ software package (SAS Institute, Inc.).

Results

ONTOGENETIC PATTERNS

All five species generally exhibited their highest μ, RNA content, P content and %P in RNA values during early larval stages and their lowest values immediately prior to pupation (Fig. 1). In general changes were relatively smooth over time, although in the cases of the slower-growing species *D. mettleri* and *D. pachea*, distinct peaks in μ, RNA content and P content can be seen, potentially corresponding to instar transitions.

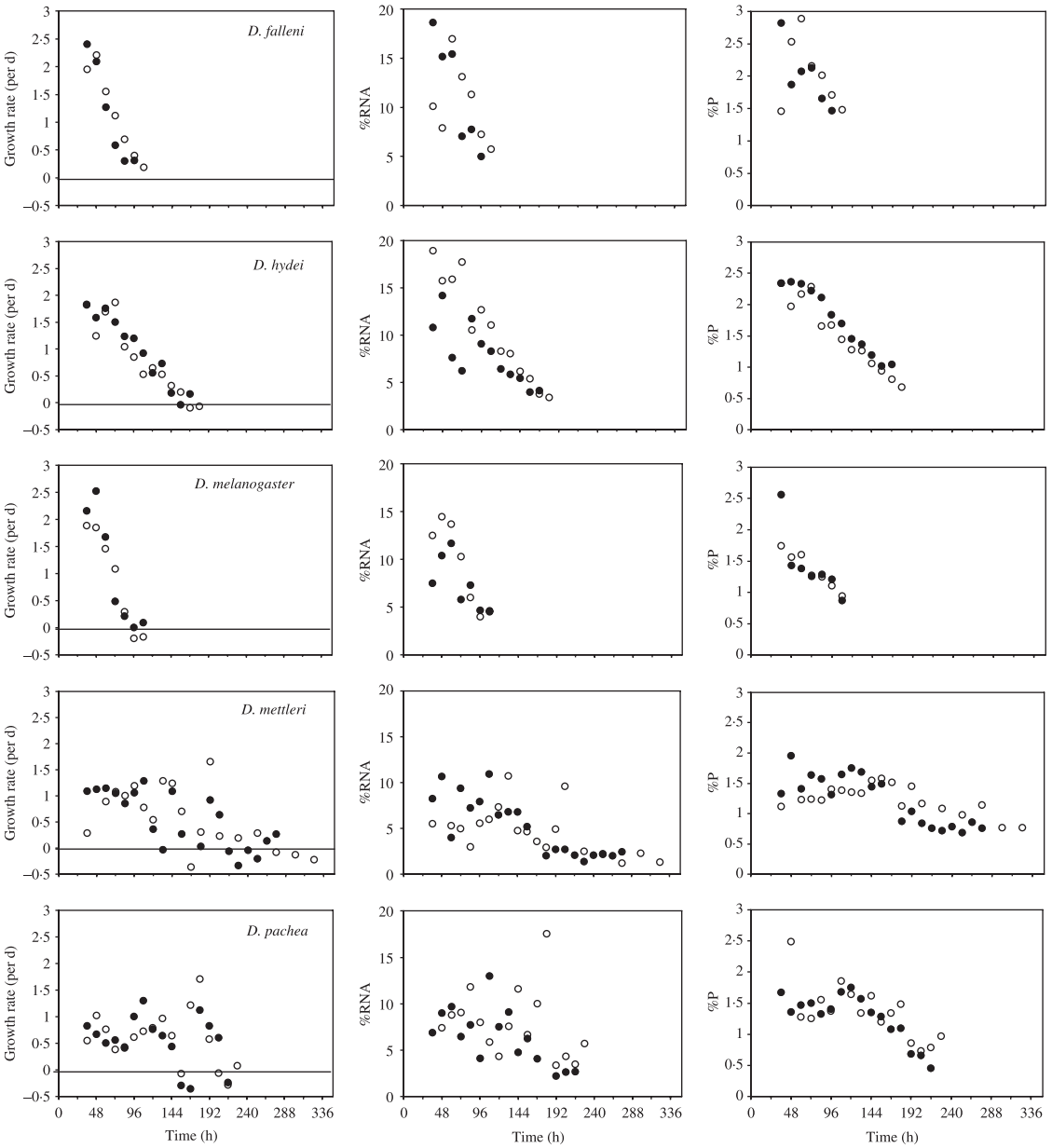

Fig. 1. Dynamics of specific growth rate (first column), RNA content (second column) and P content (third column) for the five species during larval development. Results from different experimental runs are indicated by the symbols (filled = Run A, open = Run B).

Table 1. Results of overall analysis of variance (ANOVA) examining effects of species (S), experimental run (R) and sampling time (T) during ontogeny. Two-way and three-way interaction terms are also shown. Analyses were performed for the entire data set and for data restricted to animals <250 mg (to remove possible effects of body size)

		Growth rate				%RNA			
		All data		<250 mg data		All data		<250 mg data	
	df	*F* ratio	*P* <	*F* ratio	*P* <	*F* ratio	*P* <	*F* ratio	*P* <
Species (S)	4	32·98	**0·0001**	9·95	**0·0001**	12·98	**0·0001**	6·41	**0·0001**
Run (R)	1	2·29	0·13	7·06	**0·0085**	7·18	**0·008**	4·56	**0·034**
Time (T)	1	352	**0·0001**	84·4	**0·0001**	174	**0·0001**	17·8	**0·0017**
S × R	4	0·965	0·42	2·45	**0·047**	6·55	**0·0001**	4·51	**0·0001**
S × T	4	58·1	**0·0001**	23·6	**0·0001**	25·4	**0·0001**	6·07	**0·0001**
R × T	1	2·62	0·106	12·7	**0·0004**	1·16	0·282	1·64	0·202
S × R × T	4	0·373	0·751	1·44	0·222	8·00	**0·0001**	2·21	0·069
		%P				%P in RNA			
Species (S)	4	2·97	**0·0001**	10	**0·0001**	11·5	**0·0001**	4·43	**0·002**
Run (R)	1	0·951	0·33	0·66	0·42	1·45	0·23	3·32	0·07
Time (T)	1	199	**0·0001**	32·5	**0·03**	37·7	**0·0001**	2·61	**0·006**
S × R	4	2·94	**0·021**	2·75	**0·0001**	5·71	**0·0002**	3·71	0·108
S × T	4	31·3	**0·0001**	7·26	**0·0001**	5·18	**0·0005**	1·45	0·221
R × T	1	1·43	0·233	1·61	0·21	0·0004	0·985	1·03	0·312
S × R × T	4	2·02	0·09	1·38	0·24	2·83	**0·025**	1·01	0·402

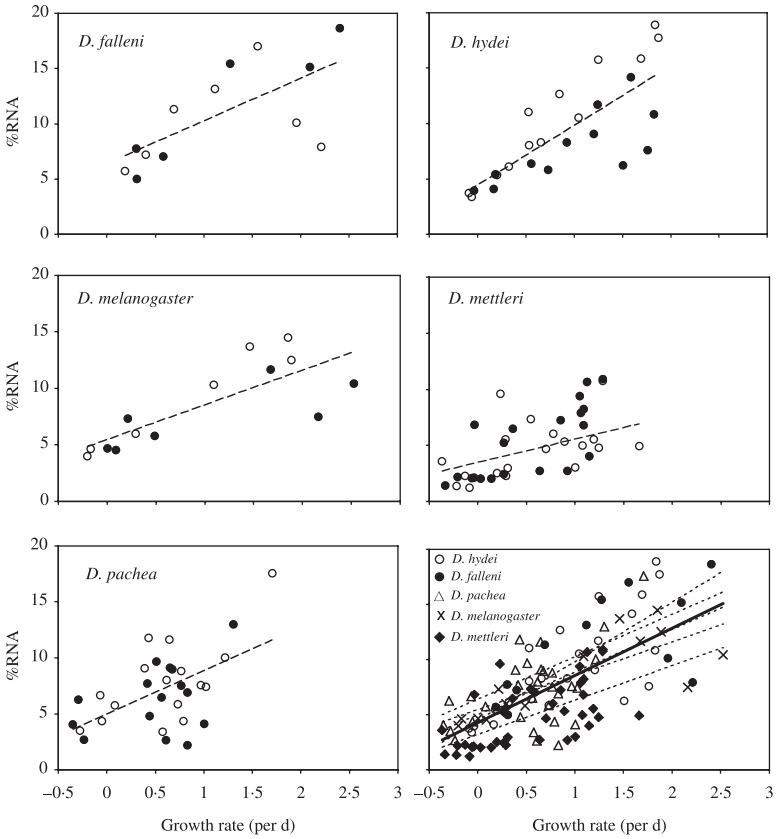

Fig. 2. Correlations of RNA content with specific growth rate for the five species. Data for the different runs are indicated by different symbols in the single-species plots as in Fig. 1. Dotted lines indicate the correlation lines fit to the data; coefficients for these relationships are given in Table 2. The final panel superimposes all the data and individual fits for each species (dotted lines) along with a relationship (dark line) for the entire data set. All relationships were statistically significant ($P < 0{\cdot}04$).

For **μ**, P content and %P in RNA, both sampling time and species were highly significant as main effects in both the overall data and in the <250 mg data set (Table 1), indicating that these parameters changed strongly during development and that the species differed overall in these parameters. Experimental run was also significant as a main effect for RNA content, along with sampling time and species (Table 1), again for both sets of data. There were multiple interaction effects for the parameters studied, especially for the <250 mg data set (Table 1). Of greatest interest is the species–time interaction, which was significant for all of the dependent variables considered. Thus, the species differed in their ontogenetic trajectories of growth and stoichiometric characteristics. This is clear in Fig. 1, in which it can be seen that three of the species (*D. falleni*, *D. hydei*, *D. melanogaster*) had high μ, RNA and *P*-values early during development that then decreased precipitously, while *D. mettleri* and *D. pachea* exhibited overall low values of μ, RNA and P that decreased slowly over an extended developmental period. The species–run interaction term was significant for RNA content, P content and %P in RNA (Table 1), indicating that experimental or (more likely) analytical artifacts may have affected the data but only for particular species in the different runs performed.

STOICHIOMETRIC RELATIONSHIPS

In general (Figs 2–4), the relationships among growth, RNA and P content were particularly strong in *D. falleni*, *D. melanogaster* and *D. hydei*, while a

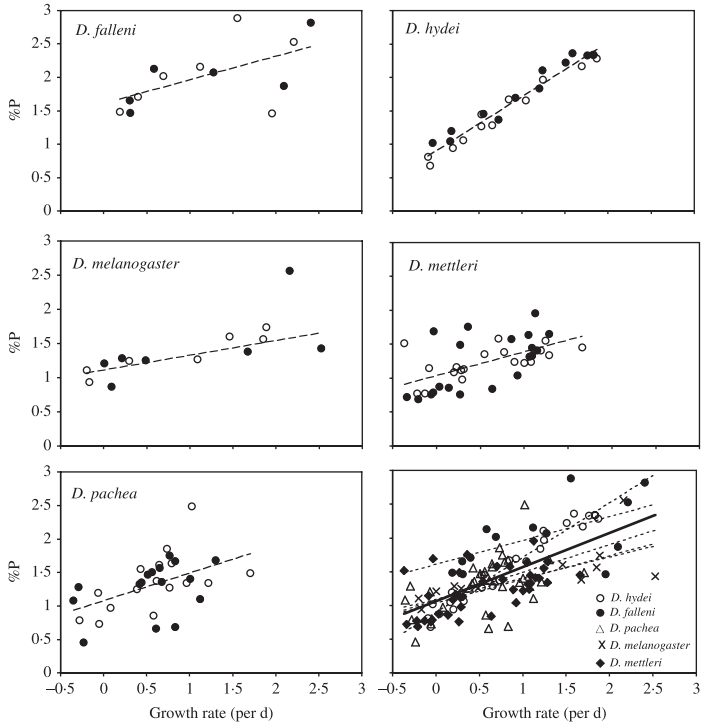

Fig. 3. Correlations of P content with specific growth rate for the five species. Data for the different runs are indicated by different symbols in the single-species plots as in Fig. 1. Dotted lines indicate the correlation lines fit to the data; coefficients for these relationships are given in Table 2. The final panel superimposes all the data and individual fits for each species (dotted lines) along with a relationship (dark line) for the entire data set. All relationships are statistically significant ($P < 0{\cdot}04$).

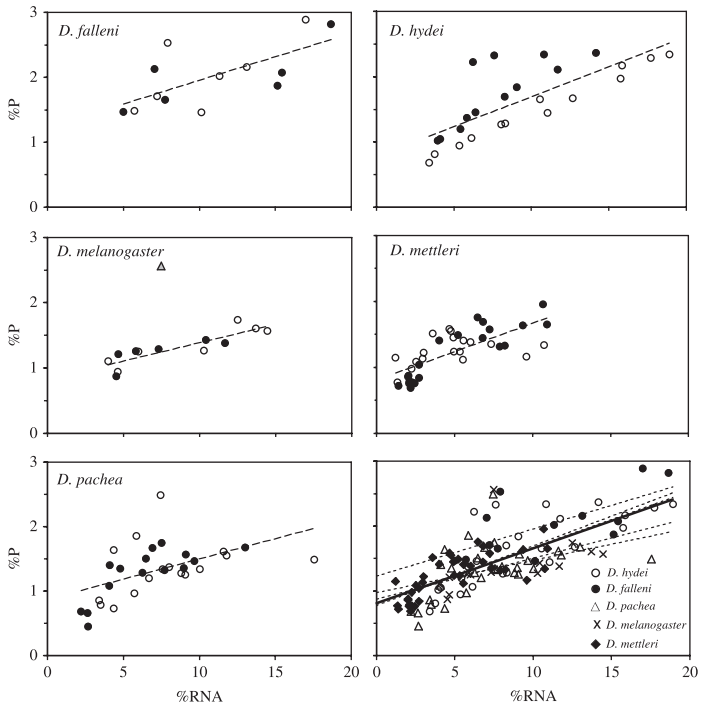

Fig. 4. Correlations of P content with RNA content for the five species. Data for the different runs are indicated by different symbols in the single-species plots as in Fig. 1. Dotted lines indicate the correlation lines fit to the data; coefficients for these relationships are given in Table 2. The final panel superimposes all the data and individual fits for each species (dotted lines) along with a relationship (dark line) for the entire data set. In the *D. melanogaster* panel, the grey triangle indicates an outlier data point that was excluded from the correlation analysis. All relationships are statistically significant ($P < 0{\cdot}003$).

substantial amount of sample-to-sample variation in RNA and P measures and of temporal variation in growth rates in *D. pachea* and *D. mettleri* (associated with instar transitions, Fig. 1) contributed to a decreased strength of correlations for these species.

Despite this, across all five species both RNA content and P content had strongly significant ($P < 0{\cdot}0001$, $r^2 \approx 0{\cdot}50$) positive relationships with μ (Figs 2 and 3; Table 2). In addition, for each of the five species considered separately, RNA content and P content were positively correlated with growth rate ($P < 0{\cdot}035$; Figs 2 and 3), although the strength of these associations differed among species (r^2 ranged from 0·24 to 0·98). Especially striking was the relationship between %P and μ in *D. hydei* ($P < 0{\cdot}0001$, $r^2 = 0{\cdot}98$). The percentage of P in RNA generally increased with growth rate (Table 2), both for the entire data set combined ($P < 0{\cdot}001$, $r^2 = 0{\cdot}18$) and for three of the study species (*D. falleni*, *D. melanogaster*, *D. mettleri*). Finally, highly significant positive correlations between RNA content and P content were observed (Fig. 4) for both the entire data set ($P < 0{\cdot}0001$, $r^2 = 0{\cdot}54$) and for each of the study species ($P < 0{\cdot}001$, $r^2 > 0{\cdot}27$). It is important to note that the slopes of these individual P *vs* RNA relationships (0·056–0·092) are close to the expected value (0·086) given that the percentage of RNA contributed by P is ~8·6% (Sterner & Elser 2002). Indeed, the slope of the relationship for the data set as a whole (0·0847 ± 0·007 SE) matches this expected value quite precisely.

The results of the GLM and ANCOVA analyses (Table 3) indicated that the species differed significantly in how variations in RNA or P content were associated with growth rate. For the overall data set, GLM analysis resulted in a significant species–μ interaction term for %P, indicating that in this set of data the species differed significantly in the slope of their %P vs μ relationships. This probably reflects the influence of the data for *D. hydei*, which had a particularly steep slope (0·81 relative to 0·2–0·4 for the other species; Fig. 3). However, for the < 250 mg data for %P, the species–μ interaction was non-significant but the effect of species was significant in the ANCOVA assuming equal slopes. Similarly, the GLM analysis for %RNA and %P in RNA involving both sets of data (the entire data set and data confined to <250 mg) indicated no significant species–μ interactions. However, in both cases the overall effect of species was significant in the ANCOVA assuming equal slopes. Thus, these results indicate that for %RNA, %P in RNA and for %P (<250 mg data only), the species differ in their stoichiometry–μ relationships owing to differences in the *y*-intercepts and not in the slopes. Finally, in both sets of data, GLM analysis examining the relationship between %P and %RNA indicated no significant species–%RNA interaction. However, the effect of species was highly significant ($P < 0{\cdot}0004$) in the ANCOVA assuming equal slopes. Thus, the

Table 2. Results of correlation analyses examining relationships among the main response variables studied. Analyses are presented separately for each species and for data for all species combined. NS = non-significant ($P > 0{\cdot}05$)

Dependent variable	Species	$P <$	r^2	Slope	Intercept
Independent variable growth rate (μ)					
%RNA	*D. falleni*	0·01	0·46	3·85	6·41
	D. hydei	0·0001	0·61	5·37	4·47
	D. melanogaster	0·0001	0·78	3·66	5·31
	D. mettleri	0·0001	0·36	3·12	3·19
	D. pachea	0·0012	0·31	3·86	5·01
	All species	0·0001	0·50	4·45	4·19
%P	*D. falleni*	0·035	0·34	0·352	1·61
	D. hydei	0·0001	0·96	0·81	0·90
	D. melanogaster	0·0011	0·64	0·215	1·11
	D. mettleri	0·0001	0·34	0·351	1·04
	D. pachea	0·004	0·24	0·409	1·08
	All species	0·0001	0·47	0·498	1·06
%RNA–P	*D. falleni*	0·048	0·31	9·97	34·5
	D. hydei	NS			
	D. melanogaster	0·018	0·38	10·87	42·4
	D. mettleri	0·005	0·19	11·0	25·0
	D. pachea	NS			
	All species	0·0001	0·18	10·32	35·2
Independent variable %P					
%RNA	*D. falleni*	0·01	0·47	0·072	1·23
	D. hydei	0·0001	0·58	0·092	0·77
	D. melanogaster	0·0001	0·75	0·056	0·82
	D. mettleri	0·0001	0·55	0·086	0·80
	D. pachea	0·0027	0·27	0·063	0·87
	All species	0·0001	0·54	0·085	0·80

species differed in the intercepts of their %P *vs* %RNA relationships.

INTERSPECIFIC PATTERNS

As noted above, GLM and ANCOVA analysis indicated that there were significant differences among species in growth rate, RNA content, P content and %P in RNA (Table 1; Fig. 5). Indeed, across the five species, species-average RNA contents and P contents were both significantly correlated with average growth rates (Fig. 6), as indicated by direct correlation analysis ($P < 0{\cdot}017$ and $P < 0{\cdot}044$, respectively) and by rank correlation analysis ($P < 0{\cdot}0001$ and $P < 0{\cdot}037$, respectively). The association between %P and %RNA was significant as a rank correlation ($P < 0{\cdot}037$) but only marginally significant as a direct correlation ($P < 0{\cdot}060$), perhaps because the relationship appeared to be non-linear (Fig. 6). The quantitative difference in average RNA across species (~5% RNA in *D. metterli vs* ~11% in *D. falleni*; difference = 6%, or 0·52% RNA–P) was sufficient to account for ~2/3 of the observed difference in average P content seen across the species (1·23% P *vs* 2·02%; difference = 0·79% P). However, there was no association between average growth rate and average %P in RNA at the species level ($P > 0{\cdot}40$ in direct and rank correlations). In sum, the interspecific results corresponded well with the intraspecific (ontogenetic) patterns: high growth rates were associated with elevated RNA and P contents while differences in RNA content were sufficient to account for differences in P content. However, unlike the intraspecific data, there was no interspecific relationship between growth rate and the percentage of body P contributed by RNA.

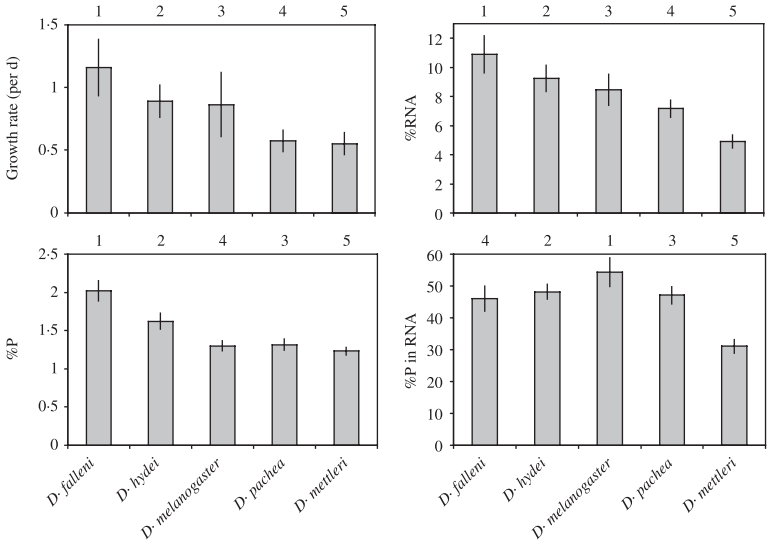

Fig. 5. Average values of growth rate, RNA content, P content and %P in RNA during the larval period for the five species studied. Numbers above each panel indicate the relative ranking (highest to lowest) for each parameter. Error bars indicate ± 1 SE.

Table 3. Results of general linear models (GLM) and analysis of covariance (ANCOVA) analyses. Analyses were performed both for the entire data set and for data restricted to animals less than 250 mg body mass. For %P (all data), GLM indicated a significant 'species'–μ interaction effect, thus indicating a difference in the slopes of %P vs μ relationships for the five species. For all other analyses, the GLM analysis indicated homogeneity of slopes and therefore ANCOVA was performed. In the ANCOVA, a significant effect of 'species' indicates that the dependent variable–covariate relationship differed among species owing to differences in the intercept. df = degrees of freedom

GLM for %P (all data)

		%P (all data)	
	df	*F* ratio	*P* <
Species (S)	4	8·84	**0·0001**
Growth rate (μ)	1	98·9	**0·0001**
Run (R)	1	0·073	0·79
S × μ	4	5·99	**0·0002**
R × S	4	0·49	0·74

ANCOVA (μ covariate)

		%RNA				% P in RNA				%P	
		All data		<250 mg data		All data		<250 mg data		<250 mg data	
	df	*F* ratio	*P* <	*F* ratio	*P* <	*F* ratio	*P* <	*F* ratio	*P* <	*F* ratio	*P* <
Species (S)	4	7·17	**0·0001**	6·43	**0·0002**	10·85	**0·0001**	3·91	**0·006**	10·56	**0·0001**
Run (R)	1	2·92	0·09	2·46	0·121	2·07	0·15	1·62	0·21	1·74	0·191
Growth rate (μ)	1	113	**0·0001**	15·1	**0·0002**	32·1	**0·0001**	4·99	**0·0001**	11·92	**0·001**
R × S	4	2·93	**0·024**	5·9	**0·0004**	4·01	**0·004**	3·73	0·057	0·4	0·8

ANCOVA (%RNA covariate)

		%P			
		All data		<250 mg data	
	df	*F* ratio	*P* <	*F* ratio	*P* <
Species (S)	4	5·76	**0·0003**	6·07	**0·0003**
Run (R)	1	1·81	0·181	4·02	**0·049**
% RNA	1	126	**0·0001**	9·91	**0·0025**
R × S	4	3·74	**0·007**	0·221	0·67

Discussion

These findings add to the accumulating data (Vrede *et al*. 2002; Elser *et al*. 2003; Gillooly *et al*. 2005), indicating that, indeed, increased growth rate carries with it a disproportionate increase in P requirements because of the need to construct P-rich ribosomal RNA, as proposed by the GRH. As in the data of Elser *et al*. (2003) involving bacteria, zooplankton and some insect species, ~40% of total biomass P in these larval Drosophilids was in the form of RNA and this percentage was directly proportional to growth rate variation during ontogeny, reaching values exceeding 90% during early larval stages with rapid growth rates. Indeed, across the entire data set, variation in P content was precisely determined by variation in RNA content after accounting for the P content of RNA (the slope of the P content *vs* RNA content relationship was 0·084, while the predicted slope due to the P content of RNA is 0·086). While patterns were strongest for intraspecific correlations, we also observed positive associations among average growth rate, RNA content and P content across the five species studied. These interspecific correlations primarily reflected differences among the species in the rate of growth (and associated RNA and P contents) during the earliest periods of development, as all five species approached zero growth rate, and relatively low RNA and P contents, immediately prior to pupation.

Before discussion of interspecific patterns, it is important to note that the five species of *Drososphila* were not raised on identical diets and thus there is some possibility that the differences among species that we observed reflected effects of diet rather than taxon-specific differences. These diets have been developed over an extended period by experts in *Drosophila* rearing and are thought to be optimal for larval growth and development of each species. We chose to use these 'optimal' diets because our study sought to compare species traits' under food-sufficient conditions

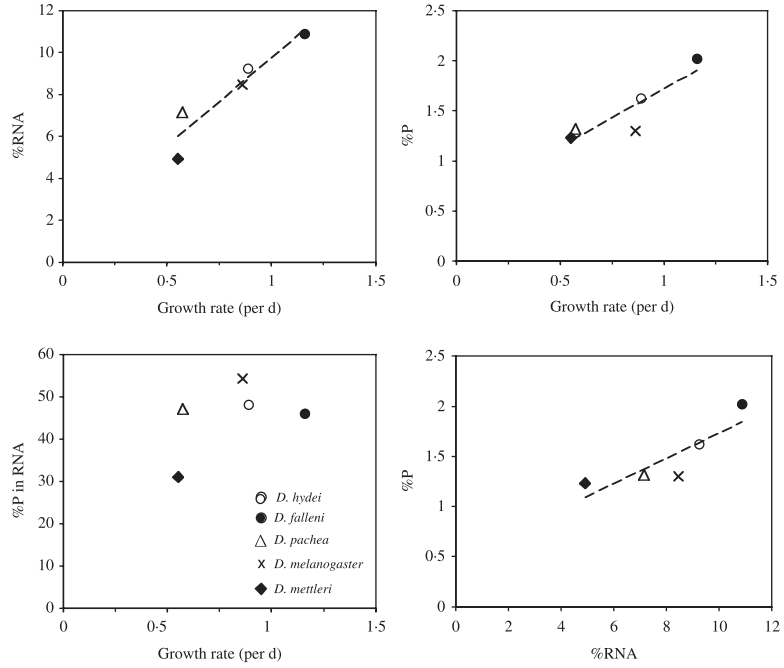

Fig. 6. Relationships of RNA content, P content and %P in RNA with growth rate and of P content with RNA content for average values for the five species studied. Values represent averages taken over the entire larval period for each species. Dotted lines indicate correlation lines fit to the data, where significant ($P < 0{\cdot}05$ for the RNA–μ and %P–μ relationships; $P = 0{\cdot}06$ for the %P–%RNA relationship).

in which each species undergoes its normal developmental trajectory. If we had chosen a common food resource for rearing all taxa (e.g. banana/*Opuntia*), it is likely that it would have been optimal for a subset of the taxa but (variably) suboptimal for others and thus we would have been confounding physiological response to suboptimal diet with species-level developmental traits. Nevertheless, the reader should keep the difference among diets in mind in considering the patterns we report. Additional studies examining possible effects of diet on larval development and stoichiometry are needed to resolve this issue. We also note that our studies involve animals obtained from populations that have been reared for at least several generations under laboratory conditions, including the media described. This may also have affected our results in that the populations may have undergone adaptive change to laboratory food and environmental conditions and thus their responses may not be completely indicative of how they perform in the wild. However, by rearing them over several generations in the laboratory rather than obtaining eggs from field-collected females, we reduce the possibility of maternal effects or other possible confounding factors that may also have influenced the data.

GLM and ANCOVA analyses indicated that the five species exhibited quantitatively similar coupling between RNA and growth rate in that the five species had similar slopes for their RNA–μ relationships (Table 3). Assuming that the overall rate of protein synthesis and retention are central in determining the observed growth rates (as assumed in the GRH; Elser *et al.* 1996, 2000b), similar slopes for RNA–μ relationships are expected if the protein synthesis capacity of individual ribosomes and the efficiency of retention of synthesized proteins are relatively uniform across species, as assumed in various models (Dobberfuhl 1999; Vrede *et al.* 2004, Gillooly *et al.* 2005; Niklas *et al.* 2005). Given this underlying uniformity of ribosome function, a similarly uniform coupling of P content with μ across species would also be expected if the growth dependence of RNA's contribution to total body P was similar across species. However, this was not the case, as the GLM analysis indicated a significant μ–species interaction for %P when all the data were considered (Table 3). This probably reflects the influence of the data for *D. hydei*, which exhibited a particularly steep slope of the %P vs μ relationship (Table 2, Fig. 3).

While the slopes of the relationships between RNA and μ and %P and μ (< 250 mg data) were uniform across species (as indicated by the non-significance of the species–μ interaction term in the GLM), there was a significant effect of species in the analysis of covariance, indicating a difference in the y-intercepts of the %RNA–μ and %P–μ (<250 mg) relationships. The *y*-intercepts indicate the RNA and P contents of each species at zero growth rate and thus can be considered to indicate each species' baseline or maintenance requirements for RNA and P. In this regard, it appears that *D. falleni*, the species with the highest average μ-value (Fig. 5) and highest maximum μ-value (along with *D. melanogaster*), has the highest *y*-intercept for both the %RNA–μ and %P–μ relationships (Table 2). This is consistent with a hypothesized trade-off (Sterner & Elser 2002) in which fast-growth rate species are at a disadvantage under slow growth conditions due to potential over-production of ribosomal RNA, as suggested for *Escherichia coli* (Stevenson & Schmidt 1998). To test this, however, it would be necessary to grow *D. falleni* at slow growth rates due to dietary P limitation and see if it maintains its higher RNA and P contents relative to other species. Such studies would be most informative if coupled to *in vitro* assays of rDNA transcription and protein synthesis rates along with assessments of rRNA and protein turnover rates under various developmental and food conditions.

Elser *et al.* (2003) presented data for RNA–P-growth coupling in a variety of heterotrophic organisms under various growth conditions. Their data suggested that there was close coupling among growth, RNA and P whenever P was likely to have been limiting to organism growth and development. When other limiting factors (e.g. energy or nitrogen intake, low temperature) were operating, the coupling was disrupted. Our data show reliable coupling among these three parameters across development in all five *Drosophila* species we considered. If the inferences of Elser *et al.* (2003) apply to our situation, then this implies the

growth rates of the larval Drosophilds in our study were generally limited by P availability. Little is known about the dietary requirements of Drosophilids with respect to P intake, nor how these relate to the composition of media normally used in rearing Drosophilids. However, it is known that the body P content of adult Drosophilids correlates positively with P-content of their dominant food source (Jaenike & Markow 2003), implying a potential role for dietary P-limitation of larval growth in shaping the evolution of life history and physiological traits in these species.

Our five-species data set is obviously too limited for a definitive assessment of possible associations between trophic strategy and patterns of growth–RNA–P coupling. However, within these limitations, it does seem that differences in the P contents of the species' dominant host foods are broadly consistent with patterns of stoichiometric coupling during ontogeny. For example, the species with the highest average and maximum μ, RNA and P contents (Fig. 5) was *D. falleni*, the mycophagous species that consumes mushrooms with very high P content (~1·5% P; data for host P content from Jaenike & Markow 2003). In contrast, both *D. pachea* and *D. mettleri* consume food materials of very low P content (0·07–0·2% P) and both exhibit extended larval periods characterized by relatively low μ, RNA and *P*-values. *D. melanogaster* and *D. hydei*, which consume foods likely to have moderate levels of available P (~0·6% P), exhibited intermediate values of μ, RNA and P. However, *D. melanogaster* did have relatively low P content (similar to those for *D. pachea* and *D. metterli*) compared with its growth rate and RNA levels. Nevertheless, these correspondences do suggest that more work examining possible associations between host food P content and μ–RNA–P coupling in *Drosophilids* is warranted.

Here we have presented data suggesting that patterns of ontogenetic development in fruit flies are associated with stoichiometric constraints presented by the dietary environment and that those associations are driven by the necessary couplings among P, RNA and growth that arise from fundamental aspects of cellular biology. The similarity of these patterns for Drosophilids to those reported for other taxa, such as crustacean zooplankton and microorganisms (Elser *et al*. 2003), suggests that reciprocal interactions between the evolution of growth rate strategies and environmental P supply may be widespread in nature, as would be expected given the broad similarity in ribosome processing across multiple taxa.

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