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Developmental responses to variable diet composition in a butterfly: the role of nitrogen, carbohydrates and genotype

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Understanding the evolution of herbivore–plant interactions requires detailed information on proximate responses to relevant dietary variability and genetic variance, if any, associated with these responses. We measured the behavioral and developmental responses of *Pieris rapae* larvae to variation in the nitrogen (N) and carbohydrate (CARB) content of chemically-defined diets, using differences in the average responses of sibling groups to estimate underlying genetic variance. Larval *P. rapae* responded to dietary reductions in both N and CARB with increased feeding, but these responses were inadequate to compensate for dietary N deficiencies within the range of N found in host plants. As a result, larvae on reduced N diets exhibited lower relative growth rates and longer development times, whereas larvae on reduced CARB diets maintained normal developmental trajectories. We also report evidence for genetic variation underlying (a) compensatory feeding responses to CARB and N availability, (b) N-driven variation in growth rate and (c) CARB-driven variation in larval duration. Our results highlight N availability as a key factor in the growth and development of this herbivorous butterfly, with CARB availability being less constraining and sufficiently addressed by changes to larval consumption rate. Furthermore, our study reveals standing genetic variance associated with larval responses to macronutrient availability, suggesting continued potential for herbivore–plant co-evolution in this system despite a putative history of strong directional selection.

The relationship between animals and their food is of profound importance to ecological and evolutionary patterns and processes. For herbivorous insects, this relationship is characterized by nutritional disparities between the needs of constructing or maintaining tissues and the nutrients available from host plants (White 1993, Raubenheimer and Simpson 1997, Sterner and Elser 2002, Schoonhoven et al. 2005). One particular imbalance, that between herbivore nitrogen (N) requirements and the N content of host plants, has received considerable attention (Mattson 1980, Scriber and Slansky 1981, White 1993). This disparity arises in part due to taxonomic differences in the structural compounds used by plants versus animals (Fagan et al. 2002). Plants primarily use carbon-based polysaccharides such as cellulose, hemicelluloses and lignin to provide structural support for their tissues (Schoonhoven et al. 2005), while animal bodies, including those of herbivorous insects, are largely constructed from protein complexes, which require high levels of N (Fagan et al. 2002, Schoonhoven et al. 2005). Accordingly, research to date has revealed a central role of dietary N availability in herbivore growth and fitness, termed 'N limitation' (Mattson 1980, White 1993, Sterner and Elser 2002).

While much of the work on N limitation in herbivores has focused on N alone, recent nutritional research has highlighted the importance of understanding the consequences of nutrient variation in food sources within a multivariate nutritional parameter space (i.e. by considering variation in several nutrients simultaneously; Raubenheimer and Simpson 1997). Considerable evidence, stemming from both ecological stoichiometry and geometric framework approaches, now supports the idea that interactions between two or more nutrient classes can have important bearing on herbivore feeding behaviors and performance (Raubenheimer and Simpson 1997, Sterner and Elser 2002).

Despite notable advances in identifying the proximate responses of many herbivores to variation in diet composition, our understanding of the evolution of herbivory is currently limited by a lack of knowledge regarding the genetic variance associated with herbivore responses. Several recent studies have indicated that genetic variance does exist in developmental parameters responsive to diet quality (Gotthard et al. 1994, Kause et al. 2001, Goverde et al. 2004), but these studies did not manipulate diet composition directly. Empirical work linking known manipulations of diet composition to patterns of genetic variation is needed to better understand how herbivore traits evolve in response to changes in plant composition.

A classic example of an 'N-limited' herbivore is the cabbage white butterfly, *Pieris rapae* (Slansky and Feeny 1977). Adult bodies of this butterfly species are ~13% N at eclosion (Morehouse unpubl.), higher in N content than any of the 152 insect species (26 lepidopteran species) surveyed by Fagan et al. (2002), and yet their host plants may naturally contain as little as 1.9% N (Slansky and Feeny 1977).

This sevenfold disparity between concentrations of N in diet and the requirements of growing larvae suggests strong selection on mechanisms for efficient uptake and processing of N from food. In addition, the ability to cope with variation in dietary N during larval development may be especially important in this species because work to date reports variable and at times maladaptive responses by ovipositing females to variation in the N content of host-plant leaves (Myers 1985, Chen et al. 2004).

Work since Slansky and Feeny (1977) has confirmed a consistently strong influence of dietary N availability on larval growth in *P. rapae* using a wide variety of host-plant species and treatments (Wolfson 1982, Gols et al. 2008, Hwang et al. 2008) as well as artificial diets (Broadway 1989, Benrey and Denno 1997, Rotem et al. 2003). These studies connect patterns of growth and consumption by *P. rapae* larvae to ecologically and agriculturally relevant variation in plant tissue composition. However, problems arise when trying to quantify the effect of N per se on developmental parameters in *P. rapae* from the work cited above. Fertilizer-induced increases in the N content of plant tissues are correlated with increases in digestible carbohydrates (Slansky and Feeny 1977) and water content (Slansky and Feeny 1977, Chen et al. 2004), as well as species-specific responses in titers of glucosinolates (Wolfson 1982, Chen et al. 2004), defensive secondary compounds produced by cruciferous plants that can be toxic to larval herbivores at high doses (Agrawal and Kurashige 2003).

Plant carbohydrate, water and glucosinolate levels are all likely to influence developmental performance variables such as growth rate, development time and compensatory feeding behaviors in ways that confound interpretation of the effect of dietary N variation alone. For example, increases in plant carbohydrate and water content are expected to result in increases in larval growth rates, which may inflate estimates of the positive effect of increasing dietary N content (Scriber and Slansky 1981, Tabashnik 1982). Likewise, variation in plant secondary compounds, including glucosinolates and protease inhibitors, is known to elicit changes in feeding behavior and post-ingestive processing efficiencies (David and Gardiner 1966, Broadway 1989, Agrawal and Kurashige 2003). For studies employing interspecific variation in plant tissue N, relationships between plant N, carbohydrates, water content and glucosinolates are even more complex (Tian et al. 2005, Nilsson et al. 2006, Gols et al. 2008, Hwang et al. 2008). Lastly, three studies that report using artificial diets with varying N contents describe dietary removals of protein without replacement with indigestible bulk (Broadway 1989, Benrey and Denno 1997, Rotem et al. 2003), suggesting that in these prior studies, decreasing N may have been correlated with increasing concentration of other nutrients (e.g. carbohydrates, vitamins, salts, etc.) and water content. As a result, the importance of dietary N availability (and its interaction with other relevant macronutrients and compounds) remains to be directly quantified in this species. Nothing is known regarding genetic variance associated with *P. rapae* responses to diet composition.

Why is understanding the influence of dietary N per se on development in *P. rapae* important? Quantifying the strength of relationships between N and larval developmental parameters is central to understanding life history evolution in this species. If N is indeed the major limiting nutrient for fitness in *P. rapae*, N may represent an elemental currency that can be used to track nutrient budgets and investment across life stages and selective contexts with respect to everything from larval predation to sexual selection on adult ornamentation. For example, male *P. rapae* invest as much as 14% of their adult N budget into pterin pigments in their wings, which generate colors that are under sexual selection via female choice, whereas females restrict their investment in wing pigments to 7% of their N budget and instead invest more N into their abdomens (Morehouse unpubl.). Sexually dimorphic patterns of nutrient investment like this are expected to be linked to differences in nutrient uptake during larval development and/or sex-linked differences in nutrient allocation strategies during adulthood. Thus, quantifying genetic variance related to N acquisition and assimilation should inform questions regarding life history evolution in this species.

We therefore sought to quantify more directly the role of dietary N availability on developmental performance and larval feeding behavior in *P. rapae*, as well as its interaction with variation in dietary carbohydrate (CARB) availability, using chemically defined diets, while holding other dietary variables constant (e.g. water content, glucosinolate titers). As a result, we can quantify the relative effects of dietary N and CARB on larval growth rate and compensatory feeding as well as on the timing of metamorphosis and on adult size. In addition, we evaluated the possibility that genetic variance underlies larval responses to diet composition by quantifying differences between sibling groups.

Based on the results of previous work, we hypothesized that *P. rapae* is 'N-limited' during larval development and therefore under strong directional selection to maximize the intake rate and assimilation efficiency of N. We hypothesized a secondary role for dietary CARB, which can influence larval growth rate or development time as a substrate for the energetic demands of developmental synthesis, but is thought to occur in dietary concentrations that more easily meet developmental requirements. In line with these hypotheses, we predicted strong effects of dietary N on all developmental parameters and on adult size and weight. We expected minor phenotypic responses to experimental variation in dietary CARB, with these responses largely restricted to the larval stage. Lastly, because we hypothesize the existence of strong directional selection on N uptake, which should reduce the associated additive genetic variance (Lynch and Walsh 1998), we predicted that genetic variation across treatments in developmental parameters would be low or non-significant.

Material and methods

Experimental animals

Larvae in this study were first generation offspring of gravid females (n = 19) collected from a wild population near Page Springs, Arizona (34°46'03"N, 111°53'37"W) from June – August 2006. These gravid females oviposited in the lab on Parafilm strips baited with cabbage leaves. We then surface–sterilized the eggs with a dilute formalin solution (Webb and Shelton 1988) before transferring them individually or in pairs to 5.5 ounce cups containing artificial

diet. For each individual, we recorded the dates of egg deposition, hatching, pupation and eclosion. All larvae were reared in a climate-controlled chamber that maintained a coincident 14L:10D photoperiod and 30°C:24°C temperature regime at a constant 55% relative humidity. Upon eclosion, adults were freeze-killed after they had hardened their wings and excreted their meconium.

Artificial diets

An agar-based holidic diet was developed by modifying a published diet for *Manduca sexta* (Ahmad et al. 1989) to approximate the nutrient content of meridic diets previously used with *P. rapae* and related species (Singh 1977, Gardiner 1985, Moore 1985, Troetschler et al. 1985). Vitamin-free casein was the sole source of dietary protein, although due to its low cystine content, we added a quantity of L-cystine to each diet. Dietary CARB was added directly as crystallized sucrose, with a known quantity of additional sucrose incorporated as part of a vitamin mix. Synthetic sinigrin hydrate was included as a feeding stimulant at 0.16 ± 0.03 μmol g^{-1} dry weight, a concentration known to elicit normal feeding on artificial diets in a related species (*P. brassicae*, David and Gardiner 1966), and within the range found in natural host plants of *P. rapae* (Nilsson et al. 2006, Gols et al. 2008). Water content of freshly prepared diet was 76.0 ± 0.6%, within the range considered optimal for larval growth (Scriber and Slansky 1981). The sealed diet containers and high relative humidity of the rearing chamber reduced evaporative water loss from the diets, but the diet was replaced in cases where diet water content was visibly reduced.

Manipulations of dietary N and CARB were accomplished by replacing casein/cystine and/or sucrose with the equivalent dry weight of cellulose, a nutritionally inert bulking agent. Nine diets were developed, representing all factorial combinations of three levels (high, mid, low) of dietary N and CARB (e.g. high N:high CARB, HNHC; mid N:low CARB, MNLC). Manipulations of dietary N and CARB were designed to mimic naturally occurring N and CARB variation in host plant tissues (Fig. 1, Table 1). To characterize variation in N availability experienced by larvae in the field, leaf tissue from wild and cultivated host plants was collected from the same field sites where we collected the female parents, freeze-dried to a constant weight, ground to a homogenous powder, and assessed with respect to the carbon and N composition using a flash combustion elemental analyzer. The N content of the final nine diets was measured using the same methods (Fig. 1, Table 1). Values for natural variation in CARB availability were derived from the literature (Table 1, Nilsson 1988, Nilsson et al. 2006).

While values from flash combustion analyses for N content are related to nutritionally accessible N (Yeoh and Wee 1994), flash combustion values for total carbon content do not correlate to CARB available for larval assimilation. Much of the carbon in plants occurs in forms that are indigestible to lepidopteran larvae, such as cellulose, hemicelluloses and lignin (Schoonhoven et al. 2005). In the artificial diet treatments, the relationship between dietary CARB and total carbon is further complicated by the incorporation of agar

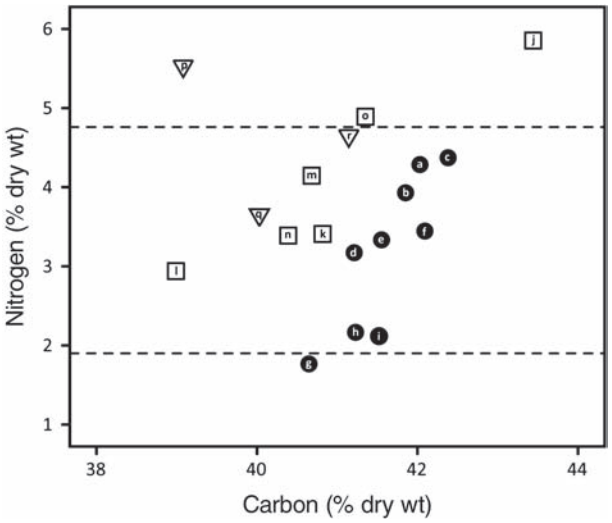

Figure 1. Nitrogen and carbon content of holidic diets (black circles), organically cultivated host plants (open boxes) and wild host plants (open triangles). Dashed lines represent the range of nitrogen contents of other *P. rapae* host plants, as reported by Slansky and Feeny (1977). Holidic diets and host plants labeled as follows (for diet abbreviations, see Methods): (a) HNHC, (b) HNMC, (c) HNLC, (d) MNHC, (e) MNMC, (f) MNLC, (g) LNHC, (h) LNMC, (i) LNLC, (j) *Brassica oleracea* var. *acephala* (collards), (k) *Brassica oleracea* var. *acephala* (kale), (l) *Brassica oleracea* var. *acephala* (cavolo nero), (m) *Brassica oleracea* var. *capitata* (green cabbage), (n) *Brassica oleracea* var. *capitata* (red cabbage), (o) *Brassica rapa* var. *ruvo* (rapini), (p) *Nasturtium officinale*, (q) *Brassica nigra*, (r) *Sisymbrium irio*.

(also nutritionally inert) and the replacement of sucrose with cellulose in mid-CARB and low-CARB diet treatments. These caveats are reflected by the lack of correspondence between dietary CARB level and total carbon content of diet treatments (Fig. 1, Table 1). Values for carbohydrate contents presented in Table 1 are a more accurate representation of experimental variation in dietary CARB.

Table 1. Nitrogen (N), carbon, and carbohydrate (CARB) content (all in % dry wt) of the nine diet treatments (for diet abbreviations, H = high, M = medium and L = low, see also Methods). N and carbon contents were measured directly via flash combustion, while CARB content was estimated from diet recipes and the level of accuracy maintained during diet preparation. Ranges for plant tissue N and carbon were derived from data reported here and Slansky and Feeny (1977); ranges for plant tissue CARB represent total digestible sugar content from Nilsson et al. (2006).

Diet	N content	Carbon content	CARB content
HNHC	4.3	42.0	30.7 ± 0.1
HNMC	3.9	41.9	22.6 ± 0.1
HNLC	4.4	42.4	14.5 ± 0.1
MNHC	3.2	41.2	30.7 ± 0.1
MNMC	3.3	41.6	22.6 ± 0.1
MNLC	3.5	42.1	14.5 ± 0.1
LNHC	1.8	40.7	30.7 ± 0.1
LNMC	2.2	41.2	22.6 ± 0.1
LNLC	2.1	41.5	14.5 ± 0.1
Host plants	1.9 – 5.9	39 – 43.4	11 – 60

Measurements

Larvae (no. of families = 13, n = 437) were split evenly across diet treatments but arbitrarily as a function of sex. The position of larval containers was randomized each day within the chamber to reduce positional effects. On day 17 post-hatching, we recorded the wet weight of each larva on a digital balance to the nearest 0.1 mg. All frass generated during each larva's first 17 days was collected, dried to a constant weight, and weighed on the same microbalance. We chose the 17 day window to maximize the length of time that larvae were allowed to feed while ensuring that measurements were all conducted prior to pupation. Larvae were then allowed to continue developing, and their pupation date recorded. We derived three larval development parameters from these measurements. Larval duration was estimated in days by subtracting the egg hatch date from the pupation date. Relative growth rate (RGR) of each larva from just before hatching (day 0) to day 17 of development was calculated as follows, where log refers to the natural logarithm (Gotthard et al. 1994):

$$\text{RGR} = [\log(\text{larval weight}) - \log(\text{egg weight})] / \text{development time}$$

Because we did not measure egg weight during the experiment, we used a published egg weight for *P. rapae* of 106 µg (Wiklund et al. 1987). Relative frass production (RFP), a measurement related to both compensatory feeding and the efficiency of conversion of ingested food to biomass, was calculated as follows:

$$\text{RFP} = \text{frass weight} / \text{larval weight}$$

Because the nature of the relationship between two variables can affect the utility of their ratio in nutritional studies (Raubenheimer 1995), we evaluated the correlation between frass weight and larval weight using standardized major axis line fitting methods (plots not shown). We find that frass weight is linearly isometric to larval weight for all diet treatments (is best described by a line fitted through the origin), and we therefore consider use of the ratio RFP appropriate (Raubenheimer 1995). In all instances where larvae shared a container, these measures were averaged for the pair and included in the analyses as the container mean.

Unfortunately, due to an outbreak of nuclear polyhedrosis virus beginning on day 25 post-hatching, most likely transferred from another laboratory stock reared in the same climate chamber, nearly all individuals from this first analysis died in the pupal stage or just prior to pupation. As a result, measures of pupal duration and adult size are lacking or incomplete for these individuals. We therefore ran a second, smaller study (no. of families = 6, n = 67) that allowed us to measure the impact of dietary N and CARB on larval duration, pupal duration and adult size. Rearing procedures and diet treatments were identical to the first study, with the exception that all larvae were reared in containers individually. Larval duration was measured as before, but we did not measure RGR or RFP for these individuals. Pupal duration was similarly defined as the number of days from pupation to eclosion. Adult weight was determined by freeze-drying eclosed individuals and weighing them to the nearest 0.1 mg on a digital microbalance. Adult size was characterized by two linear measurements: forewing length and hind femur length. Forewing length was measured using digital calipers as the distance between wing tip and attachment to the thorax of the left forewing. Hind femur length was measured for the left hind leg using a calibrated ocular micrometer (in 25 µm increments) on a dissecting microscope. Sex was determined using both sex-specific wing patterning and inspection of genitalia.

Statistics

Data were found to be normally distributed and homoscedastic, as evaluated using normal probability plots, spread–versus–level plots and Levene's test (SPSS 16.0, SPSS, Inc., Chicago, Illinois). The influence of dietary N, dietary CARB and Family on larval duration, RGR and RFP were evaluated using analysis of variance (ANOVA), with N level and CARB level as fixed factors and Family as a random factor. Container occupancy (1 or 2 larvae) was included as a fixed factor, but was found to be non-significant in all analyses (Table 2), suggesting that larval competition imposed negligible effects in co-occupied containers. For pupal duration and measures of adult size and weight, N level, CARB level and sex were included as fixed factors. However, the smaller sample size of this latter study prevented the inclusion of Family in these ANOVA models. For all analyses, full ANOVA models with all interactions were fitted first. Non-significant interactions were then removed except the experimentally-important interaction between dietary N and CARB, resulting in final models containing all main effects, the N × CARB interaction and any other significant interaction terms. Type III sums of squares were used in all instances. Results of multiple comparisons were corrected using the Tukey–Kramer method to maintain an experiment-wide α of 0.05. The above statistical procedures were conducted in SPSS 16.0 (SPSS, Inc., Chicago, Illinois). A priori statistical power analyses were conducted using G*Power 3.0.10 (Faul et al. 2007).

Effect sizes are reported as generalized omega squared statistics (ωG2), calculated from formulas in Olejnik and Algina (2003). These are unbiased estimates of the proportion of total variance in the population explained by a given ANOVA term, roughly comparable to adjusted r-squared values in regression analyses. We follow Cohen (1988) in interpreting effect sizes of 0.01, 0.06 and 0.14 as small, medium and large, respectively.

While analyzing each response variable independently provides important information, compensatory feeding, growth rate and larval development time are likely to be correlated, and for the latter two, may be causally linked (Nijhout 1981, Davidowitz et al. 2003). To better understand the relationships between these variables, we developed a structural equation model to quantify covariances amongst our three larval response variables using Amos 16.0 (SPSS, Inc., Chicago, Illinois). We further explored the relationship between relative frass production and relative growth rate under different nutrient availabilities using standardized major axis (SMA) line fitting methods, which are appropriate when both variables are known to contain equation error (i.e. biologically relevant variation unaccounted for by the bivariate

Table 2. Results from final ANOVA models for relative frass production (RFP), relative growth rate (RGR) and larval duration

Parameter	Source of variation	DF	MS	F	p	ωG2
RFP	N	2	31.165	66.55	<0.001	0.408
	CARB	2	8.523	12.06	0.001	0.149
	Family	10	2.176	2.61	0.032	0.150
	Container occupancy	1	0.306	1.35	0.247	0.001
	N × CARB	4	2.891	12.73	<0.001	0.107
	N × Family	12	0.493	2.17	0.014	0.036
	CARB × Family	14	0.820	3.61	<0.001	0.093
	Error	237	0.227			
RGR	N	2	0.324	97.60	<0.001	0.474
	CARB	2	0.003	1.50	0.226	0.003
	Family	10	0.016	5.31	0.001	0.181
	Container occupancy	1	0.000	0.14	0.705	0.000
	N × CARB	4	0.005	2.59	0.037	0.017
	N × Family	12	0.003	1.84	0.043	0.025
	Error	252	0.002			
Larval duration	N	2	484.673	34.54	<0.001	0.431
	CARB	2	26.579	1.20	0.323	0.007
	Family	5	20.003	0.70	0.637	0.000
	Container occupancy	1	6.884	0.49	0.486	0.000
	N × CARB	4	16.189	1.15	0.341	0.007
	CARB × Family	9	29.697	2.12	0.042	0.114
	Error	58	14.031			

relationship in question, Warton et al. 2006). SMA routines, including line fitting and likelihood ratio tests for common slope, were conducted in SMATR 2.0 (Falster et al. 2006).

Results

Larval development

Diet composition strongly influenced all larval development parameters (Table 2). We found large effects of dietary N, CARB and Family on relative frass production (RFP). Dietary N and CARB explained an estimated 41% and 15%, respectively, of the variance in relative frass production. Lower levels of both N and CARB in diets resulted in increased RFP (Fig. 2a–b), with a non-additive increase in RFP on the LNLC diet (significant N × C interaction, Table 2). Families differed in RFP (explaining 15% of variation in RFP), suggesting a heritable basis for larval compensatory feeding responses. Significant interactions between Family and dietary N and CARB (Table 2, Fig. 2a–b) provide further evidence that genetic variance underlies compensatory feeding responses to diet quality.

We detected large effects of dietary N and Family on relative growth rate (RGR, 47% and 18% of variance respectively, Table 2). However, variation in dietary CARB did not influence larval growth rate (Table 2, Fig. 2d). Larvae reared on mid- and low-N diets exhibited significantly lower growth rates (Fig. 2c), and this response was mediated in part by family identity (significant N × Family interaction, Table 2, Fig. 2c), although this interaction effect was relatively small. A significant but small N × CARB interaction was also found, driven by slightly different responses to dietary CARB on mid-versus low-N treatments (MNHC<MNMC<MNLC, LNHC<LNMC<LNLC).

Larval duration was strongly influenced by dietary N (43% of variance, Table 2). Larvae raised on low N diets took nearly twice as long to develop to pupation (Fig. 2e). However, dietary CARB, Family and N × C were not significant predictors of larval duration (Table 2). We did find a moderate interaction between Family and dietary CARB (Table 2) resulting from differential responses to dietary CARB between families, especially between low- and mid-CARB diets (Fig. 2f). Larval duration results from our second study were consistent with a significant effect of dietary N on larval duration ($F = 6.92$, $p < 0.01$), a minor influence of dietary CARB

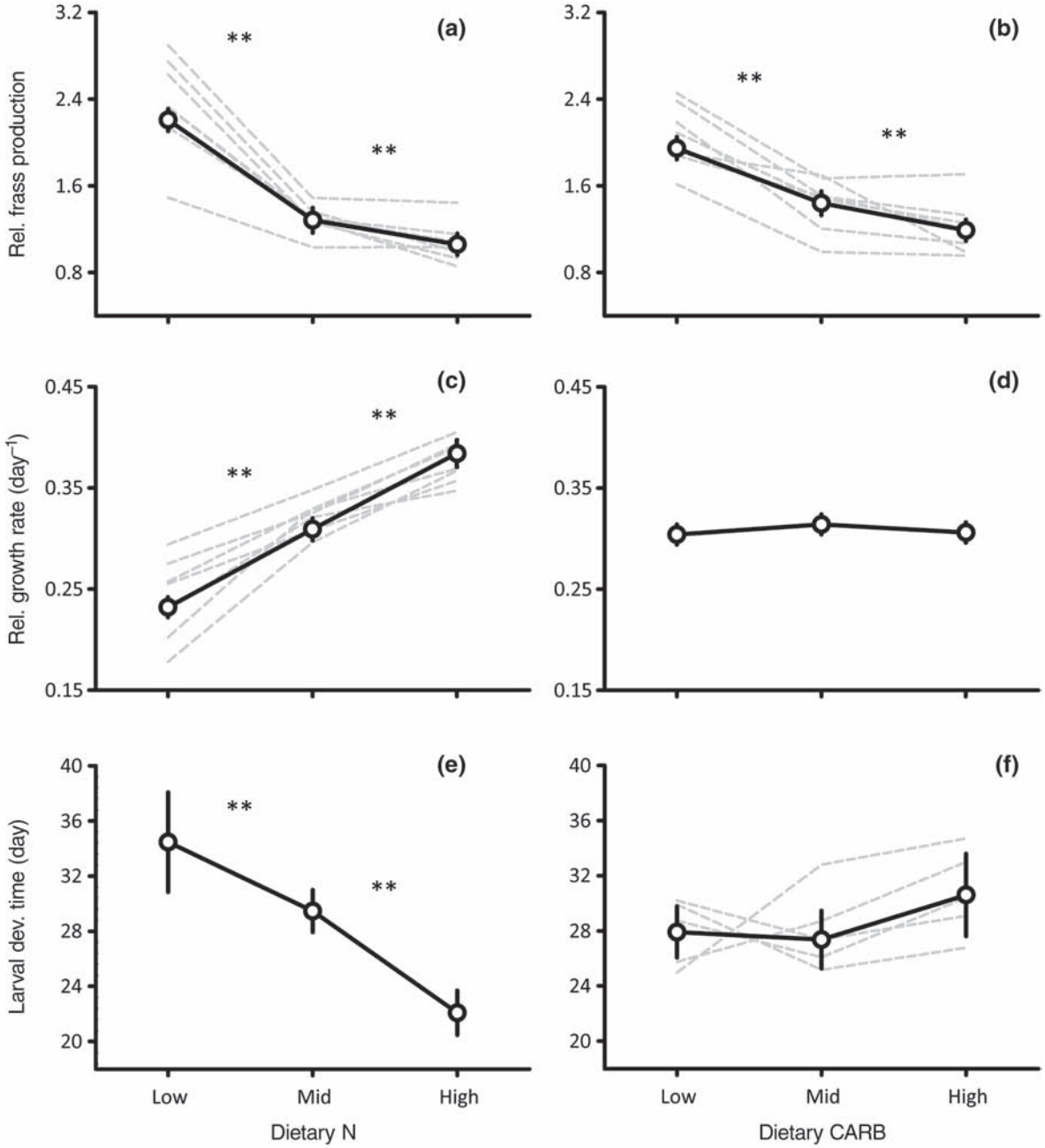

Figure 2. Effect of dietary N and CARB availability on relative frass production (a, b), relative growth rate (c, d), and larval development time (e, f). Data are estimated marginal means ± 95% CI; means that are statistically distinct are labeled with double asterisks (**). All differences in figure are transitive. Family-specific responses are drawn with dashed–grey lines in instances where a significant diet × Family interaction was found. Family reaction norms are plotted only for families with complete representation on all relevant diet treatments.

($F = 3.27$, $p = 0.05$), and a non–significant N × CARB interaction ($F = 1.63$, $p = 0.19$). Sex was also a non-significant predictor in this second study ($F = 0.49$, $p = 0.49$).

Estimates from structural equation modeling indicated, as expected, that RGR and larval duration were strongly negatively correlated (standardized regression weight = –0.942, $p < 0.001$, $r^2 = 0.88$, Fig. 3). However, RFP had no influence on larval duration directly (standardized regression weight = –0.004, p = 0.933, Fig. 3), but instead modulated growth rate (correlation coefficient = –0.513, $p < 0.001$, Fig. 3). This latter negative relationship suggests that increased frass production is associated with decreases in growth rate, which is true when viewed across all diets, because individuals on low N diets exhibit both higher frass production and lower growth rates. However, within a given dietary context, we expected frass production to positively correlate with growth rate (Slansky and Feeny 1977). As predicted, within each diet RFP and RGR were positively correlated (Fig. 4). However, the strength of the relationship varied with dietary N (Fig. 4a). Larvae on the low-N diets did not grow at rates equivalent to those on mid- or high-N diets, despite exhibiting the highest consumption (frass production) rates. Larva on mid-N diets, while closer in growth rate to larvae on high-N diets, still had a statistically shallower slope between RFP

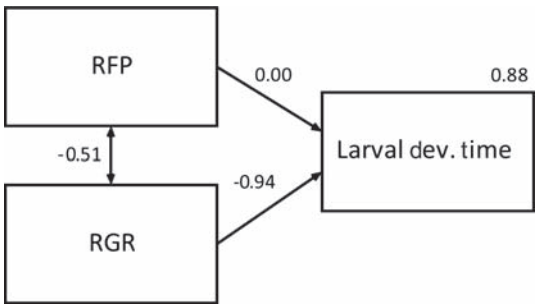

Figure 3. Structural equation model for the relationship between the three response variables. For simplicity, error terms are not displayed. Numerical labels above one-headed arrows are standardized regression coefficients; the double-headed arrow is labeled with a correlation coefficient. The numerical label above larval development time is the amount of variation in development time accounted for by the model (r^2). All estimates were derived using maximum likelihood procedures.

and RGR, suggesting that incomplete compensation occurred even on mid-N diets. In contrast, larvae achieved similar growth rates via compensatory feeding on all levels of dietary CARB, with a slightly lower slope only appearing in the low CARB treatments (Fig. 4b). This is consistent with the ANOVA findings, which indicated an effect of dietary CARB on RFP, but not on RGR (Table 2).

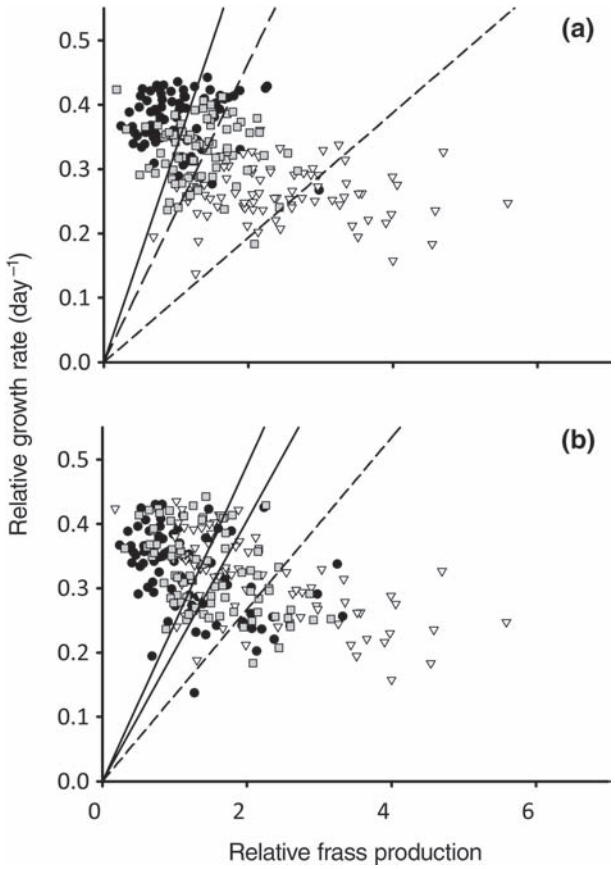

Figure 4. Relationship between relative frass production and relative growth rate, as influenced by dietary N (a), and dietary CARB (b). Best fit lines, one per level of N or CARB, were estimated using standardized major axis routines; lines that differ in stroke (solid, long dash, short dash) have statistically distinguishable slopes ($p < 0.05$). High, mid and low treatment data in both plots are represented by black circles, grey-filled squares and open triangles respectively.

Pupal duration and adult size

Despite strong effects of diet composition on larval growth and development, we detected no influence of larval diet on pupal duration, or measures of adult size and weight. All ANOVA models, from fully factorial models to reduced models including only N, CARB, Sex and N × CARB, yielded non-significant results. A priori power calculations indicate expected statistical power for reduced ANOVA models to be ~0.8 ($\alpha = 0.05$) for effect sizes similar to those found for dietary N on larval development parameters. We therefore conclude that statistical non-significance of reduced ANOVA models for pupal duration ($F_{8,67} = 1.631$, $p = 0.136$), forewing length ($F_{8,67} = 1.437$, $p = 0.201$), femur length ($F_{8,67} = 1.447$, $p = 0.197$), and adult body weight ($F_{8,67} = 1.576$, $p = 0.152$) reflects small or non-existent effects of dietary N and CARB on these parameters, suggesting that larval responses to diet quality are decoupled from adult size during metamorphosis.

Discussion

Pieris rapae has long been considered an exemplar of an N-limited herbivore (sensu White 1993). Indeed, our results demonstrate strong effects of N availability on larval development and feeding behavior in this lepidopteran herbivore. On controlled, chemically defined diets, *P. rapae* larvae responded to decreases in dietary N with increases in consumption rate (Fig. 2a), but these changes were insufficient to maintain growth rates exhibited on diets higher in N (Fig 2c, Fig. 4a). As a result, larvae on increasingly N-diluted diets required a longer larval period to attain the critical weight needed to pupate (Fig. 2e). This means that larvae on N-deficient diets not only fed more per unit time, but also fed for longer to meet their N requirements for growth, which suggests that larvae increased both the rate and total number of feeding bouts on limiting diets. These findings are consistent with effects reported from previous studies on *P. rapae* that used induced or existing N variation in plant tissues, including compensatory feeding responses (Slansky and Feeny 1977, Hwang et al. 2008), and effects on larval growth rate and duration (Slansky and Feeny 1977, Wolfson 1982, Benrey and Denno 1997, Chen et al. 2004, Hwang et al. 2008).

Larvae also responded to experimental variation in dietary CARB, but these responses were restricted to compensatory feeding behaviors (Fig. 2b). CARB-driven adjustments to consumption rate were largely independent of compensatory feeding for dietary N, with the exception of a non-additive increase in compensatory feeding on diets lowest in both N and CARB availability (Table 2). This latter interaction may indicate a threshold at which energy for growth becomes critically limiting, and cannot be supplemented by gluconeogenesis using protein sources (Thompson et al. 2003). However, compensatory feeding for dietary CARB appears to have been adequate to maintain growth rates (Fig. 2d, Fig. 4b), resulting in normal larval durations even on diets lowest in CARB. We know of no study that has addressed responses to variation in dietary CARB in *P. rapae,* but these results are at least consistent with expectations from work on other lepidopteran and non-lepidopteran insect herbivores (Raubenheimer and Simpson 1997).

Despite strong influences of diet composition on larval developmental parameters, we found no evidence for similarly large effects on pupal duration or adult size and weight. Previous measurements of these parameters in other studies on *P. rapae* have shown mixed responses of pupal duration and adult size to variation in plant N content (Wolfson 1982, Chen et al. 2004, Hwang et al. 2008). However, we find it surprising that the large effects of dietary composition on larval duration, feeding and growth rate reported here do not translate into detectable differences post-pupation. Compensatory feeding and prolongation of larval development may be sufficient to allow larvae to reach their 'growth target' (sensu Raubenheimer and Simpson 1997) prior to pupation (Berner et al. 2005). However, there are other possibilities. First, the lack of detectable differences in gross adult size and weight may conceal substantial differences in the body composition of adults reared on various dietary regimes. Large decreases in adult lipid stores and storage proteins have been reported in other insects reared under similarly limiting diets (Lee et al. 2004, Hahn et al. 2008). Second, larval mortality may have differed between diet treatments and thus biased survival towards those individuals able to best cope with experimental dietary challenges. We noted no obvious differences in mortality between diet treatments during the study, but we did not quantify mortality and therefore may have missed more subtle patterns. In spite of these caveats, our results suggest that compensation during larval development largely buffers the effects of diet quality on pupal duration and adult size and weight.

Lastly, we report preliminary evidence for genetic variance underlying compensatory feeding responses to both dietary N and CARB, growth rate responses to dietary N, and variation in larval development time related to dietary CARB (Table 2, Fig. 2). Differences between sibling groups in response to dietary N variation appear to be greatest on low N diets (Fig. 2), a pattern predicted for many traits in increasingly challenging environments (Lynch and Walsh 1998). In contrast, variation in family responses to CARB appears to be equal across dietary CARB treatments, with the shape of reaction norms differing between families (Fig. 2). While life-history traits such as growth rate and development time are predicted to exhibit higher levels of epistatic and dominance effects (Roff and Emerson 2006), our results present the possibility that additive genetic variance may mediate larval responses to dietary quality in *P. rapae*. Tissue composition of *P. rapae* host plants varies seasonally, spatially and even between tissues on a given plant (Loader and Damman 1991, Merritt 1996, Moyes et al. 2000), which may impose variable selective pressures on larval development parameters, in turn potentially maintaining additive genetic variance for responses to diet quality (Byers 2005). Indeed, significant narrow-sense heritabilities (h^2) have been reported for development time and final larval mass in sawflies grown on birch tissues of seasonally varying quality (Kause et al. 2001), and evidence for genetic variance associated with development time and growth rate has been reported for two other lepidopteran species (Gotthard et al. 1994, Goverde et al. 2004), although none of these studies manipulated diet quality directly. Our results suggest that genetic variance persists in traits associated with larval feeding and growth despite putatively strong directional selection imposed by N limitation.

What might be the proximate mechanisms involved in developmental responses to variable diet quality, and what selective pressures are likely to be operating on these underlying mechanisms? Compensatory feeding is one mechanism for coping with variable dietary resources, and we report clear evidence for compensatory feeding responses in our study. Further, we find evidence for genetic variance underlying feeding responses to both dietary N and CARB treatments. Compensatory feeding is a complex phenomenon, likely to be influenced by a wide variety of factors including chemosensory responses to food quality, the magnitude of hunger-induced motivation, and the size and frequency of feeding bouts (Simpson and Raubenheimer 1996, Lee et al. 2004). In the field, compensatory feeding responses to N and CARB may be constrained by the toxicity of co-ingested glucosinolates (Agrawal and Kurashige 2003), where glucosinolate titers may be extremely variable (Moyes et al. 2000, Tian et al. 2005, Nilsson et al. 2006). This variability presents a heterogeneous selection environment with respect to glucosinolate toxicity, which could in theory lead to the maintenance of genetic variation underlying compensatory feeding responses to dietary macronutrients.

Even with compensatory feeding, we found evidence for genetic variation in growth rate related to dietary N availability. Consumption and larval growth are related by the rate and efficiency with which nutrients are absorbed and assimilated from the diet (Raubenheimer and Simpson 1998). Nutrient absorption can be augmented in a number of ways, from changes in gut size (Yang and Joern 1994) to altered protease profiles in the gut (Broadway 1989, 1996). Recent work on amino acid transporters suggests that these might be variable amongst individuals and responsive to dietary quality (Reuveni et al. 1993, Neal 1996). In addition, naturally occurring variation in a protein kinase associated with carbohydrate acquisition has recently been identified in *Drosophila melanogaster* (Kaun et al. 2007). Again, evidence for genetic variance associated with nutrient absorption and assimilation is largely absent in the literature, but variation in nutrient availability as well as the quality (e.g. protein quality) of available macronutrients (Broadway and Duffey 1986, Felton 1996) may lead to the maintenance of genetic variation in physiological traits associated with nutrient uptake.

From an organismal standpoint, variation in the acquisition of resources from larval diet is likely to be the target of strong directional selection from a variety of sources. During larval development, reduced growth rates are predicted to result in increased predation risk, a relationship that has been empirically verified in *P. rapae* (Loader and Damman 1991, Benrey and Denno 1997). If development to pupation is successful, holometabolous insects including *P. rapae* must then allocate the resource pool acquired during larval development to adult traits (Boggs 1981). Under circumstances of nutrient shortage, trait investments are constrained, resulting in tradeoffs associated with adult fitness (Boggs 1981, van Noordwijk and de Jong 1986). Such tradeoffs may result in decreased longevity (Boggs and Freeman 2005), decreased learning ability (Kolss and Kawecki 2008), compromised immune responses (Ojala et al. 2005, Lee et al. 2006, Stoehr 2007), and reduced fecundity (Awmack and Leather 2002,

Rotem et al. 2003). Because selective pressures and adult phenotypes often differ between the sexes, nutrient requirements and associated tradeoffs may also be sex-linked (Telang et al. 2003, Maklakov et al. 2008). In *P. rapae*, a number of sex-specific adult traits are extremely resource demanding, particularly in terms of N, to which adult access is extremely limited (Boggs 2003). For males, exaggerated pterin-based wing coloration may require as much as 14% of the total adult N budget (Morehouse unpubl.). In addition, male *P. rapae* may invest as much as 28% of their adult wet weight during copulations over their lifetime in the form of protein-rich spermatophores (Bissoondath and Wiklund 1996). Females, on the other hand, invest much of their adult resources in egg-laying, a similarly resource-demanding activity supplemented to some degree by the nutrients contributed by males during mating (Karlsson 1996). Further investigation of these sex-specific nutrient dynamics and their relation to sexual and natural selection in *P. rapae* is clearly warranted.

In sum, we identify a central role for N-limitation in larval development and feeding behavior in the lepidopteran herbivore, *P. rapae*, with a relatively minor role for variation in carbohydrate availability within natural bounds. These effects appear restricted to the larval stage, although more work is needed to evaluate changes to the body composition and life-history characters of adult *P. rapae* reared on different dietary regimes. In addition, we report preliminary evidence for genetic variation underlying larval responses to dietary quality. This latter finding joins a surprisingly small set of studies that have evaluated the potential for genetic variation to mediate developmental responses to variable diet quality in herbivorous insects. Our study therefore lays a strong empirical foundation for future work on the evolution of life history characters and herbivory in this ubiquitous butterfly species.

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