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Three hundred and fifty generations of extreme food specialisation: testing predictions of nutritional ecology

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Abstract

We used a strain of diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), that had been reared for approximately 350 generations in a precisely characterised environment to test hypotheses regarding the influence of nutritional heterogeneity on the evolution of nutrient regulatory responses. Caterpillars were maintained with ad libitum access to a diet that emulated that of an extreme nutritional specialist, comprising a homogeneous food of fixed nutrient composition. We measured performance (survival, development rate, and pupal mass), as well as the protein and carbohydrate intake of individual caterpillars confined to one of a range of single foods differing in their protein, carbohydrate, and water content. In a separate experiment, we measured the amount and balance of protein and carbohydrate self-selected by caterpillars presented with nutritionally complementary foods. Results showed a close fit with three of four predictions about the nutritional responses of 'nutrient specialist' feeders: (1) survival, development rate, and pupal mass were highest for animals given diets with the protein:carbohydrate composition of the ancestral culture diet, and dropped off sharply with higher and lower protein:carbohydrate balance, (2) caterpillars coped poorly with dietary dilution by water, irrespective of the macronutrient balance, and (3) the self-selected intake point corresponded with the macronutrient balance that gave peak performance (i.e., that of the ancestral culture diet). The fourth prediction, that caterpillars would be disinclined to over-ingest nutrients on imbalanced diets, was at best weakly met. We hypothesise that the evolution and maintenance of the specialist strategy might, paradoxically, require some degree of environmental heterogeneity.

Introduction

The centrality of feeding and nutrition in animal biology leads to the expectation that mechanisms underlying the choice, intake, and post-ingestive processing of foods are subject to strong selection. In recent years, nutritional regulatory responses have been measured in a wide range of species, from insects to humans (Lee et al., 2002, 2003; Simpson et al., 2002; Mayntz et al., 2005; Simpson & Raubenheimer, 2005; Thompson & Redak, 2005; Raubenheimer & Jones, 2006; Behmer & Joern, 2008), with the broad aim of establishing a general view of how patterns of nutrient regulation correspond with key nutrition-related aspects of an animal's evolutionary environment (Raubenheimer & Simpson, 2003). This programme has been facilitated by advances in the protocols for quantifying and interpreting patterns of nutrient regulation, but it remains a considerable challenge to measure – at temporal scales that are germane to the animal – ecological variables such as the range, distribution, and composition of foods available in the evolutionarily relevant environment. While estimates of these variables may be possible for current environments, they are sufficiently difficult to obtain that examples are rare

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(Oftedal, 1991; Wright et al., 2003) and, as has been demonstrated by Crossman et al. (2005), may result in fundamental misattributions of diet. If it is difficult to assess current nutritional environments, it is at best substantially more challenging to reconstruct ancestral environments.

An alternative approach to elucidating relationships between the environment and nutritional phenotypes of animals is to begin with a well-defined ancestral nutritional ecology, from which precise predictions about nutritional regulatory responses can be generated and tested. In this paper we take such an approach, using a micro-lepidopteran culture – the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) – that had been maintained on an artificial diet for approximately 350 generations (since 1988, with 1 generation lasting 25–30 days) (Shelton et al., 1991). This laboratory culture offers a lineage that has evolved over an appreciable time in a constant and well-characterised nutritional environment, in which food is typically available ad libitum, has fixed nutrient composition, and is sufficiently homogeneous to preclude selective feeding by the caterpillars. Given that wild diamondback moths are specialists on one plant family – the Brassicaceae (Talekar & Shelton, 1993) – we had good reason to suspect that after 350 generations in the ultra-homogeneous ecology of the ancestral culture the insects in our experiments represented an extreme form of dietary specialisation. This well-defined ancestral nutritional ecology provides the basis from which we could make the following predictions:

1. Work on other insects suggests that there is a balance of protein and carbohydrate intake that is optimal, and that performance drops as the diet composition deviates from this balance (Raubenheimer & Simpson, 1997; Simpson et al., 2004). Furthermore, the steepness of the performance decline seems to be correlated with diet breadth, such that specialists experience a steeper decline as their diets move away from optimal (Figure 1). This may reflect the fact that specialists experience a more homogeneous nutritional environment compared to generalists (Simpson et al., 2002; Raubenheimer & Simpson, 2003). In the present study, we predicted that the highly selected strain of caterpillars will maximise survival, development rate, and pupal mass on diets containing the macronutrient composition of the ancestral culture diet, and that performance would decline sharply as the nutritional compositions of the test diets deviate from this composition.
2. Herbivorous insects have a documented ability to compensate by increasing ingestion when the macronutrient content of their food is diluted using water (Timmins et al., 1988; Slansky & Wheeler, 1989, 1991). However, the homogeneity of the ancestral culture diet of the caterpillars in our experiments leads to the expectation that there should be a limited capacity to process surplus water and thus to compensate for macronutrient dilution.
3. It is difficult to predict whether the caterpillars in our experiments would separately regulate their intake of carbohydrate and protein, rather than some combined quality such as diet volume. Lack of separate regulatory capacities might be expected on the grounds that the two macronutrients are present at a fixed ratio in the food and, therefore, controlling amount eaten (e.g., through use of gut stretch receptors) will serve as a perfect surrogate for both carbohydrate and protein consumption. On the other hand, because caterpillars must adjust consumption to take account of changes with development in the ratio, as well as the amounts of macronutrients needed for growth and reproduction, it might on these grounds be expected that separate mechanisms for regulating the intake and utilisation of protein and carbohydrate should exist (Raubenheimer, 2007). We predict that if the caterpillars are able to regulate their intake of protein and carbohydrate, they should select a protein:carbohydrate ratio close to that found in the ancestral culture diet (1:1).
4. Recent empirical studies demonstrate that when confined to nutritionally imbalanced foods specialist feeders have a lower capacity than generalists to ingest excesses of the surplus nutrient thereby reducing the shortfall of limiting nutrients (Lee et al., 2002, 2003; Simpson et al., 2002; Raubenheimer & Simpson, 2003; Raubenheimer et al., 2005). Accordingly, the specialised cultured lineage should show a limited ability compared with generalists to over eat the excess nutrient to gain more of the limiting nutrient in imbalanced diets.

These hypotheses were tested using the Geometric Framework, an approach developed for measuring the interactive effects of multiple nutrients on animals (Raubenheimer & Simpson, 1993, 1997, 2003; Simpson & Raubenheimer, 1993, 1995). It is, to our knowledge, the first study to test these predictions in a single species with precisely known ancestral nutritional ecology.

Materials and methods

Insects

Diamondback moth eggs were imported from Dr Anthony Shelton, New York State Agricultural Experimental Station (NYSAES, Cornell University, Geneva, NY, USA) under licence from DEFRA (PHL 225/4187) and a culture was established in the Zoology Department, University of Oxford. This Cornell strain of moths was established in

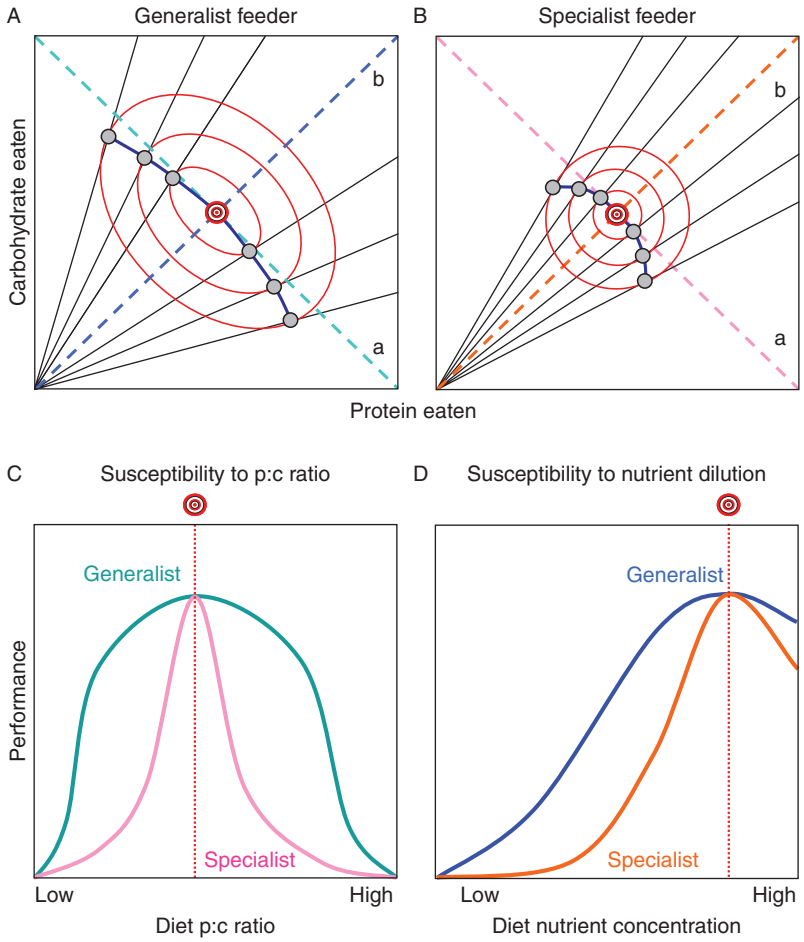

Figure 1 Schematic summarising the predicted differences between nutrient generalist and specialist feeders. In the hypothetical examples, both the generalist (A) and the specialist (B) have the same intake target (point of protein:carbohydrate intake that maximises fitness is indicated as a bull's-eye symbol). The red isoclines define the fitness landscapes for each feeding type, with a maximum at the intake target. The generalist has a tilted elliptical fitness surface, whereas the specialist's fitness surface is more restricted and circular (see Simpson et al., 2004 for theoretical basis). The generalist, compared to the specialist, is less susceptible to variation in p:c ratio in the diet. This is also shown in C, where performance is shown along a cross-section taken at line 'a' from A (turquoise-dashed) and B (pink-dashed). The generalist is also better able to tolerate dilution of dietary nutrients, as shown in D. Here performance is shown along a cross-section taken at line 'b' in panel A (blue-dashed) and panel B (orange-dashed). The grey points on each diet indicate the point of maximum fitness available when restricted to a given food rail (thin black lines). Collectively, the array of such points (the purple-curved line on A and B) defines the extent to which animals tolerate ingesting excesses and deficits of nutrients on sub-optimal diets (those that do not intersect the intake target). The intake array for the generalist is less curved than that for the specialist.

1988 using ca. 500 individuals collected at the NYSAES, and has been reared in continuous culture ever since on a wheatgerm-based meridic diet (Shelton et al., 1991) containing ca. 26% protein and ca. 26% digestible carbohydrate, taking wheatgerm to comprise 25% protein and 20% carbohydrate (Waldbauer & Battacharya, 1973). A modified version of the same diet was used for all rearing of the maintenance culture at the University of Oxford. Rearing took place in an incubator (1200-l series 4 model; LMS, Kent, UK) set at 26 °C and L16:D8 cycle.

Egg sheets were sterilised by immersion in a 3.8% formaldehyde solution for 15 min, then rinsed in water for a further 45 min. Sheets were thereafter dried under a laminar flow hood and cut into small pieces, each bearing approximately 200 eggs. Egg sheets were then transferred to 500-ml plastic tubs containing meridic diet (filled to approximately 1 cm depth and covered with a firmly attached lid). Pupae were collected prior to eclosion and transferred to 30 × 30 × 30 cm aluminium oviposition cages (BioQuip Products, Rancho Dominguez, CA, USA)

containing two small, cotton-plugged microcentrifuge tubes of 10% (wt/vol) sucrose solution. Cages contained doubled sheets of aluminium catering foil that had been dipped in a boiled cabbage solution (65 g in 500 ml) and air-dried. Each sheet was also heavily scored with forceps tips to create multiple grooves in which females would deposit their eggs.

Diets

Wheatgerm-based agar diets were prepared according to Shelton et al. (1991). In total, 10 unique diets were used. Seven of these had the same total protein (p) and digestible carbohydrate (c) level (52% by dry mass) but differed in the ratio of the two macronutrients: (1) p47:c5, (2) p40:c12, (3) p33:c19, (4) p26:c26, (5) p19:c33, (6) p12:c40, and (7) p5:c47. The remaining three diets had their total p + c diluted by 50% using water: (8) p20:c6, (9) p13:c13, and (10) p6:c20. One of the key ingredients in the Shelton diet is wheatgerm (27.1% by dry mass), which contains both protein and carbohydrate. Because of this, it was necessary to reduce the wheatgerm content of the diets to 15.8% dry mass (contributing 3.95% protein and 3.16% carbohydrate to the diet) to keep dietary protein and carbohydrate levels below 5% (hence enabling construction of diets as extreme as p5:c47 and p47:c5). To reach the level of 52% of protein and carbohydrate, casein and sucrose were added at 44.9% dry mass to each of the undiluted diets. The precise ratio of these two compounds was determined by the required macronutrient profile of the diet. Once the diets were made they were stored in a refrigerator at 4 °C until used, but never kept for more than the larval development time of a single generation (typically 6–10 days).

Experimental protocol

Performance consequences of lifetime rearing on different diets. The aim of this first experiment was to investigate the performance consequences of feeding diamondback moth caterpillars on different dietary p:c ratios from hatching until pupation. Here we used eight of the 10 diets (p47:c5 and p5:c47 were omitted because they represented extreme departures from the p26:c26 ancestral culture diet). The base of a 5-ml pipette tip was used to cut cylindrical plugs (ca. 1 cm diameter × 2 cm long) from each of the eight diets and these plugs were then each sliced in half widthways. Two 1 × 1 cm plugs of the same diet were placed into 50-mm Petri dishes containing 10 newly hatched larvae, which had originated from egg sheets taken from the laboratory culture. Each dish was then sealed with Parafilm and transferred to the incubator. Dishes were inspected daily, and every 3 days the diet was replaced. Caterpillars that pupated were removed and weighed to within 0.01 mg using a Perkin-Elmer AD-4 autobalance (Waltham, MA, USA) set at 20 mg range. Each treatment was replicated five times, for a total of 50 larvae per diet.

Choice feeding bioassays with 4th instars. To test for an actively defended point of macronutrient intake (the 'intake target'), five treatment groups of final-instar caterpillars were presented with a pair of nutritionally complementary foods, where the treatments differed in the p:c ratios of the foods in each pair. Each pairing provided larvae with an opportunity to compose a balanced diet, but to do so they had to distribute their feeding between the foods in very different ways. The five pairings were: (1) p47:c5 + p5:c47, (2) p40:c12 + p12:c40, (3) p33:c19 + p19:c33, (4) p40:c12 + p19:c33, and (5) p33:c19 + p12:c40. Given that the Cornell strain of *P. xylostella* had been reared on a diet of approximately p26:c26 for ca. 350 generations, we anticipated that its optimal intake of protein and carbohydrate would match this nutritional composition. Thus each of the five diet pairings was chosen such that the two foods spanned the likely position of the intake target.

The caterpillars used in this set of experiments were initially reared on the p26:c26 diet at 26 °C and 16:8 photoperiod, until they reached the final stages of their third larval stadium. Head capsule diameters closely follow Dyar's Rule (Robertson, 1939) and thus larval stadia could readily be confirmed. Larvae with bulging head capsules were transferred to a Petri dish containing no food and placed in an incubator where they were monitored every 2 h for ecdysis. The experiment was initiated once sufficient numbers had moulted into the fourth larval stadium.

Foods were prepared for feeding trials by using a razor blade to cut cubes weighing ca. 35 mg. This amount was slightly higher than daily food consumption but not so high that there was an inflated probability of errors in consumption estimation (Schmidt & Reese, 1986). Each block was then weighed to the nearest 0.01 mg using the autobalance set at 200 mg range. Discs of filter paper were cut to fit into the base of 35-mm Petri dishes and were moistened with ca. 0.2 ml water to maintain high humidity and prevent food blocks from desiccating. The two foods in a pair were spaced 5 mm apart in the centre of the dish. As the foods looked almost identical to the human eye, to enable their subsequent identification the food blocks were positioned with respect to a small mark made on the side of each dish. Freshly moulted larvae, contained within a gelatine capsule to prevent them crawling off the weighing pan, were then weighed to the nearest 0.01 mg and added individually to each dish. Petri dishes were next placed on trays and, before placing the trays in the incubator,

randomly rotated so that any positional biases in feeding preference could be avoided. Each choice treatment was replicated 15 times.

The two foods in each dish were replaced at 24-h intervals with freshly weighed cubes of the appropriate p:c ratio. The remains of the two food pieces were placed into individual wells in a 96-well plate, moved into a drying oven at 40 °C for 4 days, and then weighed to the nearest 0.01 mg. This food-changing procedure was repeated daily until the larvae had entered the pre-pupal stage, at which point they were given no further food. Dishes were then checked every morning and evening, and once the larva had pupated they were removed and sexed. Any cocoon material or remains of the larval head capsule were removed from pupae prior to weighing. Pupae were then killed by freezing at −20 °C, dried for 4 days at 40 °C, and weighed to within 0.01 mg. Lipids were extracted by placing dried pupae into individual wells in a Whatman Multichem™ plate and subjecting them to three 8-h chloroform rinses.

To calculate initial dry mass of food cubes used for feeding bioassays, control cubes of each food were cut, weighed, dried to constant mass, and then re-weighed. We then created regression equations for each diet based on 12 control cubes. R^2-values for each regression were never below 0.9, and in most cases above 0.95.

No-choice feeding bioassays with 4th instars. In this experiment each final instar was presented with one of seven diets differing in p:c ratio but containing the same total macronutrient concentration: (1) p47:c5, (2) p40:c12, (3) p33:c19, (4) p26:c26, (5) p19:c33, (6) p12:c40, and (7) p5:c47. Each treatment was replicated 12 times. The details of the protocol for the no-choice experiments were identical to the choice experiment, except that each 35-mm Petri dish contained only a single food.

Statistical analysis

Aspects of caterpillar performance, food, and macronutrient consumption were variously analysed by analysis of covariance (ANCOVA) and multiple analysis of covariance (MANCOVA). For analysis of intake, initial larval fresh weight was used as a covariate (Raubenheimer & Simpson, 1992, 1994). Where significant differences were detected, post-hoc comparisons (Tukey) were carried out (with α values corrected for multiple contrasts by Bonferroni transformation, as appropriate). For MANCOVA analyses, Pillai's trace statistic was chosen as the test statistic because it is considered the most robust to violations of assumptions governing parametric analysis (Scheiner, 1993). Data were checked in all cases for normality and homoscedasticity (Bartlett test). Differences in development time were tested by Accelerated Failure-Time analysis (Fox, 2001), whereas differences in survival were analysed using the χ^2-test. Analyses were performed using SAS v8.2 (SAS Institute, Cary, NC, USA) or Statview (SAS Institute).

Results

Performance consequences of lifetime rearing on different diets

Survival, development rate, and pupal mass achieved by larvae reared from hatching until pupation on each of eight diets are shown in Figure 2. The two most striking features of these results are that larvae performed markedly

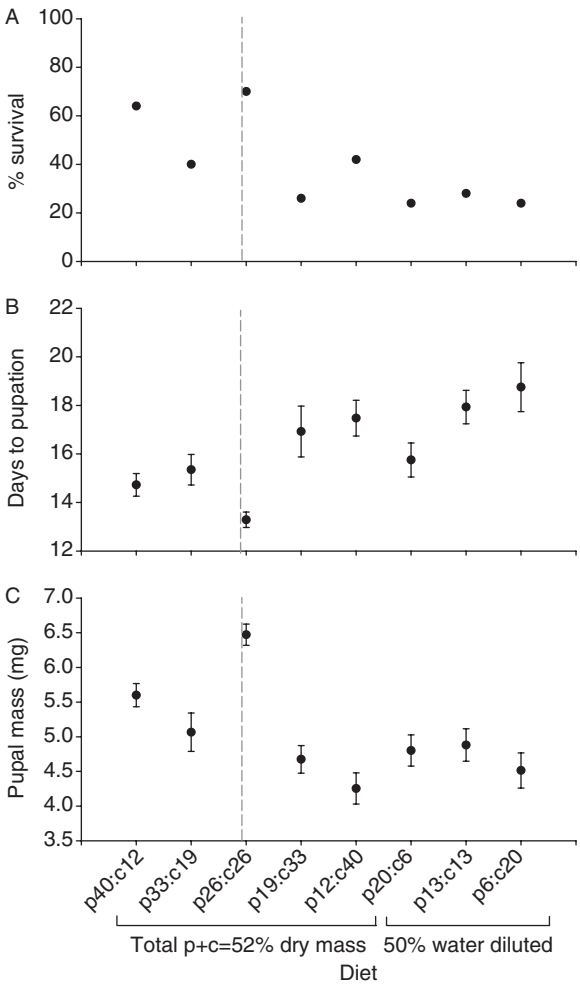

Figure 2 The relationship between dietary protein:carbohydrate ratio and performance measures of *Plutella xylostella* larvae reared on specified diets from hatching until pupation. (A) Larval survivorship on the different test diets. (B) Development time and (C) pupal mass for surviving larvae. Datapoints represent mean ± SEM for all survivors within each treatment. The vertical dashed line indicates the composition of the ancestral culture diet and hence the postulated position of the intake target.

better on the ancestral culture diet composition (p26:c26) than on any other, and that they coped very poorly with dietary dilution.

On the five undiluted diets, survival differed significantly with nutrient balance ($\chi^2 = 26.69$, d.f. = 4, P<0.001). The effect was non-monotonic, with an overall trend of decreasing survival with decreasing dietary protein being broken by a sharp peak corresponding with the protein:carbohydrate ratio of the ancestral culture diet (p26:c26; Figure 2A). On the water-diluted diets there were no differences in survival ($\chi^2 = 0.88$, d.f. = 2, P = 0.645), but survival on these diets was significantly lower than the p26:c26 ancestral culture diet ($\chi^2 = 32.62$, d.f. = 3, P<0.001).

Larval duration on the undiluted diets was significantly affected by both p:c ratio ($\chi^2 = 80.01$, d.f. = 4, P<0.001) and sex ($\chi^2 = 12.45$, d.f. = 1, P<0.001). Development time decreased as the dietary protein level rose, but was substantially shorter on 26p:26c than expected from this trend (Figure 2B). Females took slightly longer on average to reach pupation than did males (mean ± SEM, 5.5 ± 0.14 vs. 5.0 ± 012 days, respectively). On diets that were diluted by 50% using water, development time was prolonged with no significant effect evident due to macronutrient balance ($\chi^2 = 4.83$, d.f. = 2, P = 0.089).

The pattern for pupal mass resembled that for survival. For caterpillars reared on the undiluted foods, there was a significant balance effect on pupal mass (ANOVA: $F_{4,115} = 19.25$, P<0.001), with larvae fed on p26:c26 positively offset from the general trend of decreasing pupal mass with decreasing dietary protein (Figure 2C). By contrast, larvae fed the diluted diets were uniformly small regardless of the nutrient balance ($F_{2,32} = 1.36$, P = 0.272). Pupal mass did not differ significantly between sexes when fed undiluted diets ($F_{1,115} = 1.31$, P = 0.255), but female pupae were significantly heavier than males across the diluted diets ($F_{1,32} = 10\,266$, P<0.001).

Choice feeding assays with 4th instars

Bicoordinate plots of the macronutrient intake by insects provided with one of five pairings of nutritionally complementary foods are presented in Figure 3. Multivariate analysis of selected nutrient intakes indicated a significant effect of food pairing (MANCOVA: $F_{8,124} = 3.45$, P = 0.001) but not initial larval mass (MANCOVA: $F_{2,61} = 1.36$, P = 0.264). Univariate analyses revealed that the effect of food pairing was associated with carbohydrate intake ($F_{1,62} = 3.79$, P = 0.008), but not protein intake ($F_{1,62} = 2.20$, P = 0.079). Post-hoc multivariate contrasts between food pairings showed that protein and carbohydrate intake points were statistically indistinguishable across four of the five pairings, with p33:c19 vs. p12:c40 being the exception. Taken as the mean of these four converging treatments, the intake target is estimated at 3.25 mg protein and 3.00 mg carbohydrate. These values give a point that lies along a trajectory (food 'rail') equating to a food containing 26.5% protein and 25.5% carbohydrate, a balance that is extremely close to the ancestral culture diet composition of 26p:26c.

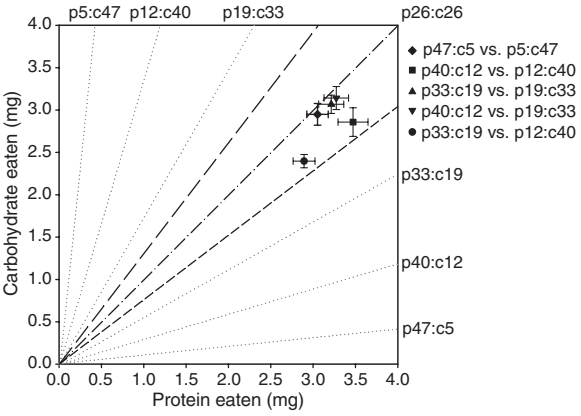

Figure 3 Bivariate mean (±SEM) for protein and digestible carbohydrate consumption by *Plutella xylostella* larvae during the choice test. The lighter, dotted lines indicate the composition of the individual diets (six in total) that made up the five unique diet pairings (shown in the figure legend). The bold, long-dashed and short-dashed lines indicate the expected proportion of macronutrients that would be ingested if caterpillars were feeding randomly on the p12:c40 vs. p33:c19 and p19:c33 vs. p40:c12 pairings, respectively. The bold dot-dashed line indicates random feeding for the remaining three food pairings.

No-choice feeding assays with 4th instars

Cumulative patterns of nutrient intake for larvae restricted to single diets are shown in Figure 4. The shape of such arrays of intake points reveals the pattern of regulatory trade-off between over- and under-ingesting nutrients by animals confined to nutritionally imbalanced foods (Simpson & Raubenheimer, 1995; Raubenheimer & Simpson, 1997), which in turn might be expected to reflect the cost structure of this trade-off (Simpson et al., 2004). Although a continuum of arrays is possible, we have defined some reference shapes which are both mathematically convenient and biologically meaningful against which to generate predictions and compare data (Simpson & Raubenheimer, 1995; Raubenheimer & Simpson, 1997). In this regard, theory has predicted, and data confirmed, that the protein:carbohydrate intake arrays for generalist-feeding herbivorous insects should approximate a line with negative slope – termed by Raubenheimer & Simpson (2003) the 'fixed proportions' (FP) configuration. The array for specialists, in contrast, should resemble more

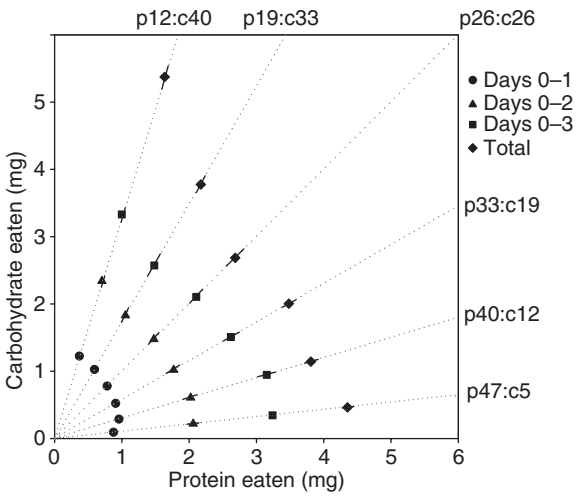

Figure 4 Mean cumulative protein and digestible carbohydrate intake by final instars of *Plutella xylostella* during the no-choice test. The dotted lines radiating from the origin indicate the composition of each diet in bivariate nutrient space, and points along each trajectory represent the cumulative intake of protein and carbohydrate over successive days. In this experiment larvae were confined to a single diet so their intake of macronutrients cannot deviate from the ratio of these nutrients present in the diet. For this reason standard errors for consumption are coincident with rails and are illustrated by way of solid lines.

closely a convex arc, termed the 'closest distance' (CD) model (see Figure 1, and Raubenheimer & Simpson, 1997, 2003).

Given that the caterpillars in our study were associated with an ultra-homogeneous nutritional environment, we anticipated that they would show an appreciably convex intake array. Figure 4 shows, however, that the array was at best moderately curved, and only so over the 1st day where nutrient intake from the two most protein-rich diets lagged behind what was otherwise a discernibly linear pattern. Across subsequent days these points moved outwards, falling better into line with the linear portion of the curve. To further investigate the degree to which these patterns corresponded with the specialist-associated CD model, we plotted against diet the mean ± SE values of the variable:

$$In_{(obs)} - In_{(t)},$$

where $In_{(t)}$ is the target coordinate for nutrient n and $In_{(obs)}$ is the observed intake point for each nutrient (Raubenheimer & Simpson, 1997) (Figure 5). This value assumes 0 for both coordinates where animals achieve the target intake, is negative for nutrient shortfalls, and positive for excesses. Also plotted is the curve showing the prediction under the CD model. This analysis shows that over the period day 0–1 the high-protein side of the array corresponded with the prediction under CD, but caterpillars on

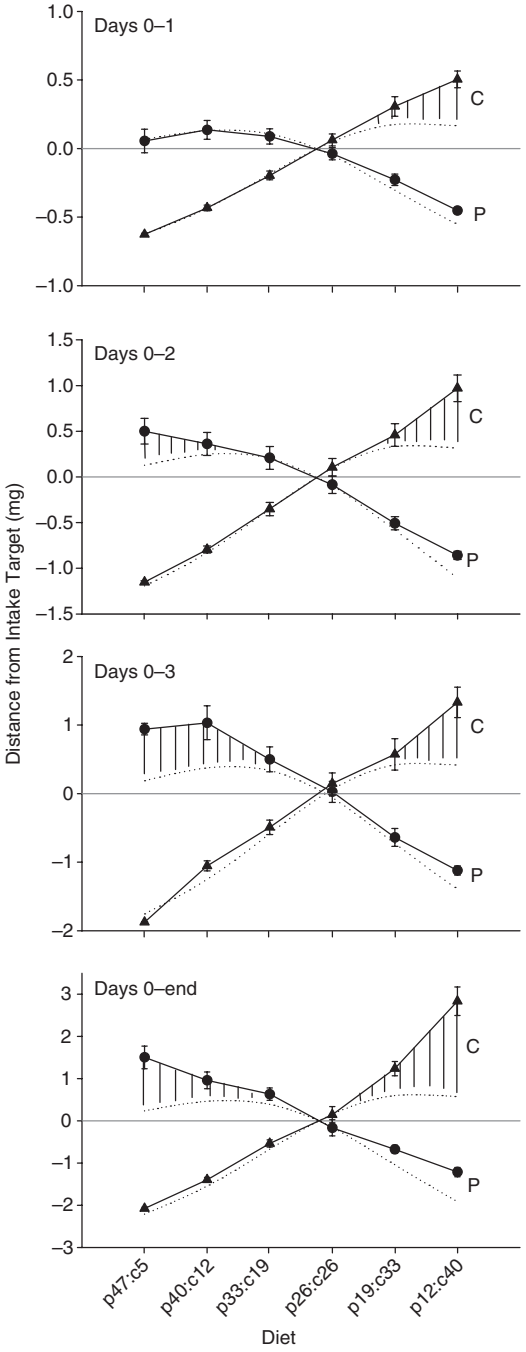

Figure 5 Error plot (Raubenheimer & Simpson, 1997) highlighting the relationship between overeating one nutrient (protein, P, or carbohydrate, C) and undereating the other nutrient vs. food composition, relative to the selected intake point (IT) from the choice test. The dotted line indicates the closest distance (CD) model that has previously been associated with specialist feeders. This intake pattern is plotted by calculating the distance from the IT to a perpendicular point on each of the diet rails. The hatched area indicates consumption in excess of that expected under the CD strategy.

the high carbohydrate diets ingested a greater relative surplus of carbohydrate, as expected under the generalist-associated fixed proportions rule (Raubenheimer & Simpson, 2003). Over subsequent days the larger-than-predicted surplus of carbohydrate intake was maintained, whereas the surplus of protein intake increased beyond the model for CD, thus approximating that expected of a generalist. Overall, therefore, our prediction that the caterpillars in our experiment would show patterns of nutrient balancing characteristic of specialist feeders was not met.

Discussion

The Cornell diamondback moth strain had been reared on a wheatgerm-based meridic diet for an estimated 350 generations prior to entering our experiments, offering the opportunity to test hypotheses about the relationship between the degree of heterogeneity in the nutritional environment and the evolution of nutritional regulatory systems. We began with four predictions, each one founded on previous experimental and theoretical work.

The primary prediction, that the caterpillars should show peak performance on the ancestral culture diet, was met spectacularly. There was a general trend for larvae to perform better (survive, develop quickly, and achieve a high pupal mass) as the ratio of protein to carbohydrate in the diet increased from p12:c40 to p40:c12, but sitting atop this baseline trend were substantially higher performance values for larvae reared on the diet with the same macronutrient balance as used in the long-term culture (p26:c26).

It could be argued that this result might reflect the formulation of a diet that supports peak performance, rather than evolutionary adaptation of the insects to perform optimally on the culture diet. Although we certainly agree that a criterion for the formulation of rearing diets is good performance, there are three reasons that make it highly improbable that this is the explanation. First, it is no trivial task to formulate diets that align multiple performance peaks as tightly as was observed in our experiment, and it is only recently that the requisite techniques have become available (Ruohonen et al., 2007); in contrast, selective processes like Darwinian evolution are well-suited to multiple optimisation problems of this sort (Cheverud, 1996; Wagner & Altenberg, 1996). Second, we know of no other insect that responds as sensitively to variations in macronutrient balance as did the caterpillars in our experiment. Such sensitivity is unlikely to occur in naturally adapted herbivores, because even specialist plant feeders encounter significant variation in the nutrient composition of their diets (Mody et al., 2007), and might on this basis be expected to show some tolerance to variations in macronutrient balance. It is, however, less surprising to see such sensitivity in a population with a long history of feeding on reliably uniform synthetic foods. Third, previous studies have demonstrated adaptive evolution of laboratory strains of insects (Carpenter & Bloem, 2002). Indeed, we have previously demonstrated that the same strain of *P. xylostella* used in the present work can adapt to dietary shifts within as little as eight generations (Warbrick-Smith et al., 2006). Overall, therefore, we are confident that the sharp performance peak that we have observed reflects a history of adaptation to extreme nutritional homogeneity.

Also in line with our predictions was the sensitivity shown by the caterpillars in our experiments to dietary dilution. Many animals, insects included, are able to ameliorate the impacts of dietary dilution by increasing the rates of food intake and/or digestive efficiencies. The African migratory locust (*Locusta migratoria* L.), for example, is able to maintain nutrient intake and performance over a five-fold range of dilutions using indigestible cellulose (Raubenheimer & Simpson, 1993), and other grasshoppers are similarly able to compensate in this way (e.g., Yang & Joern, 1994). Caterpillars, too, have been found to compensate for dilution of their foods either by cellulose or water (Timmins et al., 1988; Slansky & Wheeler, 1991; Lee et al., 2004). In contrast, the caterpillars in our experiments showed dramatically reduced performance with 50% dilution by water, even on the diet with balanced macronutrient content (p13:c13) (Figure 2). This inability to cope with variation in the water content of foods likely reflects a history of feeding on homogeneous foods with near-constant water content.

A third notable result was the close fit between the balance of macronutrients selected in the choice experiments and the balance that gave optimal performance. Such a close fit has previously been observed in caterpillars (Simpson et al., 2004) and locusts (Raubenheimer & Simpson, 1997), and it does not seem unreasonable to expect that this will always be the case excepting circumstances where traits are constrained by adaptive trade-offs or tested within evolutionarily unfamiliar environments (Raubenheimer & Simpson, 2007). Indeed, there is an extent to which nutritional homeostasis (the position of the intake target) will passively follow selected changes in performance peaks, and not necessarily rely on separate evolutionary changes. This is because nutrient regulation is directly linked to the levels of specific macronutrients in the haemolymph, and haemolymph composition is influenced by the rate at which nutrients are withdrawn by cells for functions such as growth, reproduction, and metabolic fuel use (Simpson & Raubenheimer, 1993) – in the words of Barton Browne (1995), feeding is 'demand-driven'.

However, there are also components of regulatory systems that are likely subject to natural selection in specific environments (Simpson & Raubenheimer, 1996), but more-detailed behavioural analyses are needed to test whether these fit the expectations for the caterpillars in our study (Simpson, 1994).

A central prediction that inspired this work was the expectation that 350 generations of extreme nutrient specialisation would result in a reduced capacity to ingest excesses of surplus nutrient in order to increase their gains of limiting nutrients. In the geometric framework, this corresponds with the prediction that the insects would show a pronounced expression of the CD regulatory pattern, as opposed to the FP pattern (Raubenheimer & Simpson, 2003). The rationale behind this expectation is that generalists encounter wider variation in their nutritional environment than do specialists, and so might be expected to have evolved physiological (e.g., storage, excretion) and behavioural (e.g., complementary feeding) means of coping with or even capitalising on surplus nutrients ingested in imbalanced foods (Simpson et al., 2002; Raubenheimer & Simpson, 2003). Although this prediction has previously been borne out in a number of comparisons between generalist- and specialist-feeding insects, support from the present study is, at best, weak: the cumulative intake arrays over days 1, 2, and 3 were only marginally curved, and then only on the high protein diets.

As is often the case for functional predictions in biology (Kacelnik & Cuthill, 1987), a poor fit between expectation and data can provide heuristic benefits. In the present case, it suggests the possibility that in making our prediction we might have over-stressed the factors that positively select for the FP strategy (tolerance and benefits of surplus nutrients), while neglecting the costs to specialists of ingesting surpluses of specific nutrients when exposed to nutritionally imbalanced foods. In this context, the present data might reflect the fact that in the nutritionally homogeneous ancestral environment the caterpillars were not exposed to the potential for ingesting deleterious excesses, and were thus subject to no selection pressures for maintaining the CD strategy. The failure to limit the excess ingestion of nutrients might, indeed, have been an important contributor to the steeply reduced performance observed for caterpillars confined to nutritionally imbalanced foods (Raubenheimer et al., 2005).

Similar logic could explain our previous demonstration that this line of caterpillars significantly accumulates body fat when exposed to high-carbohydrate foods (Warbrick-Smith et al., 2006), whereas it might arguably be predicted that in the energetically secure environment of the ancestral culture there was no selection pressure for accruing lipid reserves (Witter & Cuthill, 1993; Speakman et al., 2004). However, as in our prediction for the intake array, it might be that the observed accrual of body fat was due to a lack of selection in the constant ancestral environment for the oxidative mechanisms required to void excess ingested macronutrient (Zanotto et al., 1997; Trier & Mattson, 2003). Interestingly, the threshold for lipid storage responded to selection within four generations of being exposed in laboratory experiments to manipulated environments with either high or low mean energetic content, although in both treatments there was heterogeneity in the available foods (Warbrick-Smith et al., 2006).

Thompson & Redak (2005) similarly observed a generalist-associated strategy in the facultative specialist caterpillar *Manduca sexta* (L.). This they attributed to the fact that *M. sexta* is not an obligate specialist. Interestingly, however, the caterpillars in Thompson & Redak's (2005) experiments were derived from stock that had been cultured for many generations on semi-synthetic foods, raising the possibility that these animals, like those in the present experiments, showed the generalist regulatory pattern because of a history of extreme dietary homogeneity.

Our study has focussed on the association in caterpillars of nutritional homogeneity and macronutrient regulation and performance outcomes. These gross-level characteristics are of course underpinned by a complex suit of mechanistic attributes, such as the form and extent to which wild-type sensory, digestive, and metabolic processes are maintained over generations. For example, an artificial diet will place very different demands upon digestive enzymes, transport proteins, metabolism of dietary molecules, and detoxification of secondary metabolites (e.g., Bowers & Puttick, 1988; Zangerl & Berenbaum, 1993; Jongsma et al., 1995). It would be interesting to learn how such factors relate to the results of the present study.

In overview, this study has confirmed three of our predictions: that the self-selected macronutrient balance would correspond with the composition of the ancestral culture diet, that peak performance would be maximal on this diet, and that the caterpillars would not cope well with dietary dilution. A fourth expectation, that the caterpillars would show a limited ability to over-ingest excess nutrient in imbalanced foods (i.e., intake array would be curved), was only weakly met, suggesting that in deriving our predictions we might have omitted one or more important considerations. An interesting possibility is that a degree of nutritional heterogeneity is necessary for the evolution of both specialist and generalist strategies, with the two phenotypes being associated with different degrees of heterogeneity. This would make the seemingly paradoxical prediction that the response to nutritionally imbalanced foods of the wild progenitor population would be more

specialist-like than the descendents which were cultured on an invariant diet. Unfortunately, the nutritional responses of the progenitor population, or of any wild population of *P. xylostella*, to artificial foods are unknown, and difficult to test because the majority of wild-caught *P. xylostella* will not feed on the meridic diet and require a period of several generations of laboratory rearing on diet to establish a vigorous breeding population. This, in itself, attests to the adaptability of *P. xylostella* to the nutritional environment, but precludes any direct comparisons with the insects in our study. A complementary approach would be to compare the responses of populations reared on differently manipulated artificial nutritional ecologies, as in Warbrick-Smith et al. (2006). It would, however, be feasible to do so only for a small proportion of the time over which the caterpillars in our experiment were exposed to the relevant nutritional ecology. However, various populations are reared worldwide on a variety of synthetic foods differing in p:c content and thus testing these strains within the context of the geometric framework would provide valuable insights into the generality of diet-adapted colonies. The fact that previous studies have found strain-by-diet interactions for DBM larval rearing (Carpenter & Bloem, 2002) strengthens the case for such work.

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