Does Bertrand's rule apply to macronutrients?

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It has been known for over a century that the dose–response curve for many micronutrients is non-monotonic, having an initial stage of increasing benefits with increased intake, followed by increasing costs as excesses become toxic. This phenomenon, termed Bertrand's rule, is widely assumed not to apply to caloric macronutrients. To date this assumption has been safe, owing to the considerable methodological challenges involved in coaxing animals to over-ingest macronutrients in a way that enables the effects of specific food components to be isolated. Here we report an experiment which overcomes these difficulties, to test whether the second phase (incurring costs with excessive intake) applies to carbohydrate intake by the generalist-feeding caterpillar *Spodoptera littoralis*. The results showed that excess carbohydrate intake caused increased mortality, thus extending Bertrand's rule to macronutrients.

Keywords: nutrient imbalance; nutritional costs; foraging theory; macronutrients; hormesis

1. INTRODUCTION

It has long been appreciated that the health and performance benefits (e.g. growth, reproduction, longevity) to consumers of some nutrients vary non-monotonically with concentration: at low levels the benefits increase with intake towards an optimal plateau, beyond which there are increasing costs as the regulatory mechanisms are overwhelmed and excesses become toxic. This relationship was mathematically formalized by French nutritionist Gabriel Bertrand (1912), into what has come to be known as 'Bertrand's rule'. Although the parameters of the function are specific to particular nutrients, Bertrand's rule is believed to apply to all essential micronutrients (Mertz 1981).

In contrast, it is widely assumed that Bertrand's rule does not apply to caloric macronutrients (carbohydrates, lipids and amino acids). Since these can be converted into usable energy, the first phase (where benefit increases with gain) is believed to apply, but not the second (plateau) or third (toxic) stages. This view underpins a large body of literature in foraging theory, where the maximization of energy gain is considered the primary goal of foragers (Sih & Christensen 2001). It also has a basis in the theory of physiological regulation. Diamond & Karasov (1987) and Karasov & Hume (1997), for example, predict that the sign of the regulatory responses of intestinal nutrient transporters for different categories of nutrients should vary. The transporters for energetic nutrients such as sugars and amino acids, which can provide calories in direct proportion to the amounts eaten, should be up-regulated with dietary concentration, whereas the activity of transporters for 'essential yet potentially toxic nutrients not used for calories (e.g. vitamins and trace minerals), should decrease monotonically with dietary substrate level' (Diamond & Karasov 1987, p. 2244).

In recent years, however, suggestions have emerged that Bertrand's rule may be more general, applying equally to caloric and non-caloric nutrients. Most conspicuous are the now well-established relationships among caloric budgets (intake–expenditure), obesity and human health (Bjorntorp 2001; Hill *et al.* 2003; Simpson & Raubenheimer 2005). Evidence for non-human animals is derived largely from studies of the patterns of ingestive regulation of macronutrients. Thus, when locusts are confined to nutritionally imbalanced foods that contain an excess of macronutrients (protein and digestible carbohydrate) relative to micronutrients (minerals), they avoid ingesting excess macronutrient even though this involves suffering a deficit of micronutrients (Trumper & Simpson 1993). If Bertrand's rule applied only to micronutrients, the opposite would be expected—i.e. locusts should accept a caloric surplus in order to avoid a micronutrient deficiency. Likewise, when confined to foods containing an excess of digestible carbohydrates relative to protein, several insects and some vertebrates are known to regulate so as to achieve both a moderate excess of carbohydrates and a deficit of protein (including essential amino acids) (Raubenheimer & Simpson 1997; Lee *et al.* 2002, 2003). If Bertrand's rule did not apply to caloric nutrients, it would be expected that protein intake should be optimal, irrespective of the magnitude of the carbohydrate excess.

Suggestive as they are, the patterns of nutrient regulation provide only indirect evidence for the applicability of Bertrand's rule to caloric nutrients. This is because it is a functional rule (i.e. it pertains to fitness benefits), so a direct test would involve measures of the consequences of caloric excesses. Such evidence is, however, difficult to obtain for reasons illustrated in the above experiments on insects; animals will not readily ingest caloric excesses (as in the experiment involving minerals), and when they do it is only a moderate excess whose consequences are difficult to separate from simultaneous deficits of other nutrients (e.g. in the protein versus

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carbohydrate experiments). Excesses could be enforced using invasive techniques (such as gavage, intravenous injections, etc.), but this involves stressing the animals to an extent that would preclude meaningful measures of performance. What is needed is an approach that coaxes animals to voluntarily ingest caloric excesses, as do humans (Simpson *et al.* 2003; Simpson & Raubenheimer 2005), thus enabling direct measures of the fitness consequences.

Here we report experiments in which we reasoned partly from first principles (knowledge of the physiological regulatory mechanisms) to design a nutritional environment in which caterpillars would voluntarily ingest a caloric excess whose effects can be disentangled from the deficits and excesses of other nutrients. Our results revealed that, as in humans, there are fitness costs to caterpillars of ingesting excesses of carbohydrates. This demonstrates for the first time that Bertrand's rule applies to macronutrient intake by a non-human animal.

2. MATERIAL AND METHODS

(a) *Insects and diets*

This study was designed both as a component of a larger experiment (Simpson *et al.* 2004) and specifically for the analysis reported here. *Spodoptera littoralis* caterpillars came from a culture at the NERC Centre for Ecology and Hydrology, Mansfield Road, Oxford, and were reared on a wheat-germ based semi-artificial diet until they moulted into their ultimate stadium (sixth). Individual insects were then weighed to the nearest 0.1 mg (obtaining initial fresh mass), and each was placed into its own experimental arena, a 90 mm diameter Petri dish that had five 1 mm-diameter perforations in the upper lid. The experimental arenas with animals were kept in an incubator (LMS Ltd, Kent, UK) set at 27 °C with a 12 : 12 light : dark photo regime. Preparation of synthetic foods was based on the method described in Simpson & Abisgold (1985). The micronutrient content comprised, by dry weight: Wesson salt (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and a vitamin mix (0.2%; Dadd 1961). The protein content was composed of a 3 : 1 : 1 mixture of casein, peptone and albumen, while digestible carbohydrate was sucrose. All foods contained the same mixture of 1 : 5 protein-to-carbohydrate, which in the larger experiment (Simpson *et al.* 2004) was shown using both self-selection treatments and performance measures to be carbohydrate-biased relative to the optimal balance (1.2 : 1) for sixth stadium *S. littoralis*. The foods differed, however, in their concentrations of the macronutrient mix, comprising either 42% (35%C+7%P), 63% (52.5%C+10.5%P) or 84% (70%C+14%P) by dry mass of the two nutrients. The gradient of macronutrient concentration was generated by altering the content of the bulking agent, alpha-cellulose (Sigma Co.), which is not digested or absorbed by caterpillars. In contrast with macronutrients, the concentration of essential micronutrients was maintained as constant across the four diets at 4% of dry mass. The low-, medium- and high-nutrient diets thus contained 54, 33 and 12% cellulose, respectively. The granular base diets were thoroughly mixed into a 1% agar solution, at a ratio of six parts agar solution to one part dry ingredients. This final step ensured that the physical consistency of the diets was appropriate for caterpillar feeding.

(b) *Dietary treatments*

Each insect was given a pair of diet blocks, with the treatments differing in the concentration of the macronutrient mix within these diet blocks ($n=10$ caterpillars per treatment). The food combinations were chosen to give a between-treatment gradient in the average macronutrient concentration to which insects were exposed. In the 'low' treatment, both diet blocks contained the protein : carbohydrate mixture at 42% by dry weight, giving an average concentration of 42%. In a 'medium-low' treatment each insect received one diet at concentration 42% and the second at 63%, giving an average macronutrient concentration of 52.5%. The 'medium high' treatment comprised diet blocks of 42% and 84% (giving an average of 63%), while the 'high' treatment comprised diet blocks of 63% and 84% (average 73.5%). These food pairings were selected partly on the basis of our experience with macronutrient regulation in *S. littoralis* (Lee *et al.* 2004; Simpson *et al.* 2004), and partly on existing knowledge of the interplay of mechanisms involved in feeding regulation in insects (Simpson 1995).

One important aspect of the design is that in order to ensure that a significant excess of carbohydrate was ingested, the experimental foods had a moderately (35%) to extremely (70%) high concentration of this nutrient. As discussed by Simpson *et al.* (2004), foods with very high concentrations of nutrients might be less phagostimulatory than more moderate foods (Simpson & Raubenheimer 1996), but might nonetheless result in the ingestion of excess nutrient because they elicit lower levels of inhibitory feedback from stretch receptors in the gut per unit of nutrient eaten than do less-concentrated foods (Simpson 1995). In an attempt to moderate any acute effects of the highly concentrated foods, we provided the insects in these treatments with a second, less concentrated food. Another reason for pairing the foods as we did, was that it provided combinations of foods with different macronutrient : micronutrient ratios, thus enabling us to generate variance in micronutrient intake that was orthogonal to the variance in macronutrient intake.

We anticipated that the insects would eat significant amounts of both the high- and low-nutrient foods in each pair. This expectation was based on previous experiments with caterpillars and other insects, and also on theoretical grounds (Simpson & Raubenheimer 1996; Raubenheimer & Jones *in press*). It has been suggested that such apparently inappropriate food selection might be adaptive sampling, which provides a means to gather information about the available foods (Westoby 1977; Greenwood 1984; Sherratt & Harvey 1993; Day *et al.* 1998). Alternatively, it might reflect sensory or cognitive constraints (Simpson & Raubenheimer 1996). Whatever the reason, we succeeded in our aim of coaxing the caterpillars to spread their intake across the paired foods: the proportion of total intake from the less concentrated of the two foods was 0.5 ± 0.11 (highest), 0.27 ± 0.14 (medium high) and 0.37 ± 0.11 (medium low).

(c) *Experimental protocol*

The newly moulted sixth instar caterpillars were weighed to the nearest 0.1 mg and subsequently assigned to one of the four treatments. During the experimental period, individual insects received two food blocks, which were weighed to the nearest 0.1 mg before being presented to the caterpillars. Amounts provided ensured that caterpillars had *ad libitum* access to the two foods throughout, but the surplus was minimal thus reducing the error of intake estimates

(Schmidt & Reese 1986). Feeding dishes were sealed with a strip of Parafilm to prevent desiccation of the foods. After each 24 h period, remaining food was removed from the arena and replaced with fresh blocks. Removed blocks were dried to constant mass at 50 °C and weighed to the nearest 0.1 mg. This procedure was repeated daily until each caterpillar ceased to feed prior to pupation. Food consumption was calculated as the difference between initial dry food mass (estimated from initial fresh food mass using regression equations) and final dry food mass. The consumption of sugars, proteins and micronutrients was calculated as the product of dry mass of each food eaten and the concentration the relevant nutrient in the food. Stadium duration was measured to the nearest 6 h from pupation. Pupae were then weighed to the nearest 0.1 mg.

(d) Performance

Performance measures were caterpillar growth over the sixth stadium, the duration of the stadium, and mortality, all of which are key components of fitness in Lepidoptera (Honek 1993). For an approximation of fitness which includes all three variables, we computed the index: [growth (mg)/development time (days)] $\times$ survival (%).

(e) Statistical tests

Effects of diet nutrient concentration on intake, the fitness index, development time and growth were tested using one-way ANOVA. To test whether the time to death differed from the stadium duration of surviving caterpillars, we performed two-way ANOVA with diet as a fixed factor and outcome (died versus survived) as a random factor. All ANOVAs were preceded by a test for equality of variances (Levene's test), and in no case was this assumption violated. A χ^2 -test was used to compare mortality rates across the treatments.

3. RESULTS

The effects of dietary treatment on intake and the compound fitness index are presented in figure 1. Caterpillar performance declined with increasing macronutrient density of the experimental treatments (figure 1a; $F_{3,23}=17.1$, $p<0.0001$ using ANOVA). This effect could not be accounted for by micronutrient intake, which was constant across treatments (figure 1b; $F_{3,34}=1.05$, $p=0.382$). Excessive cellulose intake can affect the performance of caterpillars through its influence on digestion and absorption (e.g. Lee *et al.* 2004), but this cannot explain the current results where performance was better at high levels of cellulose intake (figure 1c; $F_{3,34}=38.2$, $p<0.0001$). By contrast, carbohydrate intake increased with increasing macronutrient density of the treatment pairings ($F_{3,34}=18.6$, $p<0.0001$), as did protein intake ($F_{3,34}=18.6$, $p<0.0001$) but with shallower slope (figure 1d). A key difference between the trends for carbohydrate and protein, however, is that the increase in carbohydrate intake was over-and-above the required (target) intake (indicated in the figure by the horizontal dashed line), and thus represented an increasing excess. Protein intake, on the other hand, was below the target level (horizontal solid line), and the increased intake on macronutrient-dense foods thus represented a diminishing deficit. Since reduced protein deficit is, if anything, likely to enhance performance, the observed

Figure 1. Effects of dietary macronutrient concentration on (a) performance (development rate $\times$ survival), (b) micronutrient intake, (c) cellulose intake and (d) macronutrient intake by sixth stadium *S. littoralis* caterpillars. In (d), the solid horizontal line represents the level of protein intake which was self-selected by caterpillars over the same developmental period and provided maximum performance (i.e. the target intake), while the dashed horizontal line represents the target intake for carbohydrate (Simpson *et al.* 2004). Symbols for nutrient concentrations are: L, lowest; ML, medium low; MH, medium high; H, highest.

Figure 2. Effects of diet macronutrient content on (a) mortality, (b) development time and (c) growth of *S. littoralis* fed one of four diet pairings. Symbols for nutrient concentrations are: L, lowest; ML, medium low; MH, medium high; H, highest.

reduction in performance could only be due to excessive sugar intake.

Figure 2 shows the raw variables that were used to compute the fitness index. Mortality increased significantly with increasing diet macronutrient content (likelihood ratio $\chi^2_3 = 14.0$, $p = 0.003$). By contrast, there was no significant effect of diet treatment on development time ($F_{3,23} = 0.103$, $p = 0.958$) or growth ($F_{3,23} = 0.048$, $p = 0.986$) of the survivors through the sixth larval stadium. A two-way ANOVA with diet and outcome (died versus survived) as factors showed that the mean time to death (7.8 ± 0.21 days) was marginally but significantly longer than the mean stadium duration for those animals that survived (6.8 ± 0.13 days; $F_{1,2.103} = 44.17$, $p = 0.019$), with no treatment $\times$ outcome interaction ($F_{2,23} = 0.326$, $p = 0.725$; this analysis excluded the low nutrient treatment, since none of those animals died).

The above analyses of intake were performed on cumulative data up to the fifth day of the larval stadium, which across all treatments represented $88.9 \pm 1.63\%$ of total intake (there was no between-treatment difference in this proportion: $F_{3,34} = 0.338$, $p = 0.798$). Basing the analysis on this subset of data avoids the possible confounding effects of performance influencing intake, when our primary interest is in the effects of nutrient consumption on performance—for example, extended development time might cause an increase in sugar intake, rather than result from it. To test the robustness of our conclusions, we also performed the tests on total intake across the sixth larval stadium. In no case did the results differ qualitatively from those reported above. Furthermore, the outcome was unchanged even when we excluded from the analysis of intake those caterpillars that died. We are therefore confident that the relationship we have observed is due to excessive carbohydrate intake influencing performance, rather than altered performance influencing carbohydrate intake.

4. DISCUSSION

Our data demonstrate that ingested excesses of a specific macronutrient can be detrimental to the performance of caterpillars, thus showing for the first time, to our knowledge, that Bertrand's rule applies to macronutrient intake by non-human animals. One reason that this has not hitherto been demonstrated is the methodological challenges involved, a problem recognized by Gerber *et al.* (1999) and Stevenson & Sielken (2000). These challenges arise in part from the fact that macronutrients exert strong leverage over the patterns of food ingestion (Raubenheimer & Simpson 1997), and as a consequence it can be difficult to induce animals to spontaneously ingest excesses whose effects can be partitioned from excesses and deficits of other food components.

In the experiment reported here, however, the strong tendency of caterpillars to regulate their intake of macronutrients was regarded not only as the problem, but also the principal means of solution. By selecting an appropriate balance of macronutrients for the experimental foods, we were able to use the leverage over intake of some nutrients to manipulate the ingestion of others. In particular, the strong tendency of *S. littoralis* to show compensatory increases in consumption of food containing low levels of protein (Lee *et al.* 2004) was used to induce over-ingestion of carbohydrates.

However, in addition to protein, the intake of carbohydrate is also strongly regulated by *S. littoralis* (Lee *et al.* 2004), as in other insects (Raubenheimer & Simpson 1999; Simpson & Raubenheimer 2000; Raubenheimer & Jones in press). For this reason we were unable to induce the caterpillars to ingest a sufficiently large surplus of carbohydrate that there was no deficit in protein intake, thus leaving open the possible confounding situation that the observed performance effects could be due both to a surplus of carbohydrate and a deficit of protein. The effect could, nonetheless, be attributed to carbohydrate, on the basis that the pattern in protein intake represented a diminishing deficit rather than an increasing surplus relative to the intake target.

It is partly for this reason—namely to distinguish ingested excesses and deficits—that identifying where the

intake target lies is a critical part of our methodology (Simpson & Raubenheimer 1995). This was achieved for each macronutrient by challenging caterpillars to regulate their intake of both protein and carbohydrate in the face of five different complementary food pairings, which they did with remarkable success, and then showing that the regulated intake point maximized performance (Simpson *et al.* 2004).

In some instances, however, measures of the target levels of food components are not available, as was the case for micronutrients in the present experiment. In this case our approach was to use the levels of macronutrients to manipulate an intake of micronutrients that did not vary with animal performance, thus experimentally controlling for the micronutrient content of the diet. For cellulose the situation was different again. As is true for several species of herbivorous insect, *S. littoralis* maintains stable or near-stable performance across a wide range of cellulose intakes (Simpson *et al.* 2004), principally because cellulose is neither digested nor absorbed and so relative to nutrients has reduced scope for exerting a metabolic influence on these animals. High levels of ingested cellulose can, nonetheless, reduce the conversion efficiencies of macronutrients (Lee *et al.* 2004). However, the sign of the across-treatment gradient in cellulose intake ruled this out as an explanation for the observed performance effects, because performance improved with increasing cellulose intake.

Our findings provide an interesting parallel to a topical issue in toxicology. Until recently it was generally accepted that the most fundamental shape of the dose-response curves for toxins is linear (increasing toxicity with dose), or threshold (toxic only beyond a threshold intake). It is, however, becoming increasingly clear that for many toxins the relevant shape is an inverted 'U' or a 'J', wherein substances that are toxic at high levels of intake are beneficial to health at low levels—a phenomenon known as 'hormesis' (Calabrese & Baldwin 2003). Like linear and threshold models in toxicology, traditional maximization models of macronutrient gain are monotonic, assuming that fitness benefits accrue in proportion to intake. The present findings demonstrate that a hormesis-like biphasic curve is more appropriate, since ingested excesses of carbohydrate are associated with decreased fitness in *S. littoralis*.

This is not the first attempt to reconcile the toxicological concept of hormesis with nutrition. Indeed, Bertrand's (1912) rule was formulated against a history of toxicity experiments on plants, yeasts and bacteria (Calabrese & Baldwin 2000). Several terms have since been suggested to generalize the biphasic dose-response curve observed in toxicology and nutrition, including 'subsidy-stress gradient' (Parsons 1992), 'toxicity-essentiality paradox' (Katz 1995) and 'nutrient-toxin dosage continuum' (Gerber *et al.* 1999). While most attention has been on micronutrients, attempts to reconcile macronutrient intake with hormesis have focused almost exclusively on the phenomenon of caloric restriction, where slowed aging has been associated with reduced energy intake (e.g. Turturro *et al.* 2000). However, this reconciliation has suffered from a lack of three essential controls in the relevant experiments (Stevenson & Sielken 2000): (i) a measure (or even an appropriate concept) of the baseline energy intake, which is necessary to determine

whether the observed benefits of caloric restriction are really due to restricted intake in the treatment groups, or excessive intake in the controls; (ii) attempts are seldom made to partition the effects of different diet components, thereby determining whether the observed responses really are due to calorie intake, and if so, whether the source of calories (carbohydrates, amino acids, lipids) matters, and (iii) most experiments rely on a single level of restriction, rather than multiple doses, and the dose-response function is thus derived by extrapolation.

The state-space geometrical approach that we have taken was designed with these problems in mind. The first control is established using a treatment in which subjects self-select their preferred intake, and a response surface is used to verify the beneficial consequences of this (Simpson *et al.* 2004). As noted by Gerber *et al.* (1999), results of toxicology experiments are particularly convincing when the experimental animals actually seek out the optimal level of the agent, as did the caterpillars in our experiments (Simpson *et al.* 2004). Second, as discussed above, in the geometrical approach the effects of different nutrients can be partitioned by manipulating orthogonally the levels of food components, and measuring the amounts of each that are eaten. Finally, the use of a graded series of experimental foods enables a comprehensive description of the dose-response relationships to be derived. We are currently using this approach to test the relationships between nutrient intake and aging in adults of *Drosophila melanogaster*.

The finding that non-human animals will spontaneously ingest carbohydrate excesses that result in adverse fitness consequences has significant implications for foraging models. It not only calls into question the traditional assumption of energy maximization, but also provides an important step towards elaborating alternative models. 'Eating to requirement' models, for example, predict that in the absence of constraints (e.g. on food availability), an animal's intake will be sufficient to meet the requirements set by its genetic potential, and no more (Emmans & Kyriazakis 2001; Yearsley *et al.* 2001). Implicit in this is the assumption that there are costs to over-eating, which are usually assumed to be extrinsic costs such as the time and energy spent foraging, risk of predation, the ingestion of parasites, etc. Our data suggest that there are also more direct, physiological costs to over-ingesting macronutrients. The key challenge now is to extend such studies to natural environments, identifying the constraints under which animals forage in the wild. This will reveal the range on the U-shaped hormetic resource curves over which animals actually forage, thus contributing to an ecological-evolutionary explanation for the emerging generality of this phenomenon (Parsons 1992; Gerber *et al.* 1999).

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