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MALE COCKROACHES PREFER A HIGH CARBOHYDRATE DIET THAT MAKES THEM MORE ATTRACTIVE TO FEMALES: IMPLICATIONS FOR THE STUDY OF CONDITION DEPENDENCE

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Sexual selection is a major force driving the evolution of elaborate male sexual traits. Handicap models of sexual selection predict that male sexual traits should covary positively with condition, making them reliable indicators of male quality. However, most studies have either manipulated condition through varying diet quantity and/or caloric content without knowledge of specific nutrient effects or have correlated proxies of condition with sexual trait expression. We used nutritional geometry to quantify protein and carbohydrate intake by male cockroaches, *Nauphoeta cinerea*, and related this to sex pheromone expression, attractiveness, and dominance status. We found that carbohydrate, but not protein, intake is related to male sex pheromone expression and attractiveness but not dominance status. Additionally, we related two condition proxies (weight gain and lipid reserves) to protein and carbohydrate acquisition. Weight gain increased with the intake of both nutrients, whereas lipid reserves only increased with carbohydrate intake. Importantly, lipid accumulation was not as responsive to carbohydrate intake as attractiveness and thus was a less-accurate condition proxy. Moreover, males preferentially consumed high carbohydrate diets with little regard for protein content suggesting that they actively increase their carbohydrate intake thereby maximizing their reproductive fitness by being attractive.

KEY WORDS: **Diet choice, *Nauphoeta cinerea*, nutritional geometry, sex pheromone, sexual selection.**

Sexual selection has been recognized as a major force driving the evolution of elaborate male traits for well over a century (Darwin 1871; Andersson 1994; Coyne and Orr 2004). A central prediction underlying handicap models of sexual selection is that male sexual traits should covary positively with condition (Andersson 1986; Pomiankowski 1987; Grafen 1990; Iwasa and Pomiankowski 1994; Andersson and Iwasa 1996), allowing male sexual traits to serve as reliable indicators of male quality in female mate choice (Johnstone 1995; Lailvaux and Irschick 2006) and male–male competition (Maynard Smith and Harper 2003; Lailvaux and Irschick 2006).

Condition is narrowly defined as the amount of resources an organism has available for allocation to fitness-enhancing traits (Rowe and Houle 1996; Lorch et al. 2003; Hunt et al. 2004; Tomkins et al. 2004). To date, condition measures have largely focused on individuals' energy reserves (i.e., fat storage) or their ability to convert these resources into somatic growth or energy (Tomkins et al. 2004). Consequently, condition is frequently manipulated by varying diet quantity and/or caloric content (Cotton et al. 2004). Many of these studies have shown that the expression of sexual traits is dependent on the amount and quality of diet available (Andersson 1994; Cotton et al. 2004; Lailvaux and Irschick 2006). Studies of the effects of manipulating the amount of carotenoids in a diet on male sexual trait expression and attractiveness have provided evidence that specific nutrients may independently affect sexual trait expression (Olson and Owens 1998; Cotton et al. 2004). However, simply manipulating the total nutrients available, or one specific nutrient, does not account for the effect that any interaction between nutrients may have on the expression of condition-dependent sexual traits or how the exact amount of each nutrient ingested influences these traits (Morehouse et al. 2010). The amount of food ingested (i.e., resources acquired) may differ greatly from the amount of resources in energy reserves, as resources may be incorporated directly into fitness-enhancing traits (Tomkins et al. 2004). Furthermore, little is known about whether males are able to preferentially consume specific nutrients to increase their reproductive success (but see Maklakov et al. 2008 for a study that found a male diet preference which did not maximize their reproductive performance).

Nutritional geometry (also known as the Geometric Framework, Simpson and Raubenheimer 1993) provides a powerful tool to examine how the acquisition of specific nutrients influences the expression of traits and fitness measures. Artificial diets are constructed to vary in both the concentration and ratio of nutrients. The intake of nutrients from these diets is then measured, along with the traits and fitness variables (i.e., fitness proxies or components, Hunt and Hodgson 2010) of interest. Response surface methodologies (Lande and Arnold 1983) can then be used to examine the independent effects of each nutrient and any possible interactions they may have on trait expression. Nutritional geometry thus provides a novel condition proxy (i.e., the amount of nutrients ingested) which can then be directly related to trait expression. By using a proxy of condition that is measured before the distribution of resources into fitness-enhancing traits (such as fat storage and pheromones), we eliminate the possibly confounding effects of individual differences in allocation to these traits (Tomkins et al. 2004) and thus employ a more accurate measure of the pool of resources available to an individual. Furthermore, feeding trials that give individuals a choice between diets can then be used to examine the regulatory behavior of dietary intake (Simpson and Raubenheimer 1993; Simpson et al. 2004).

In this study, we use nutritional geometry to examine the effects of protein and carbohydrate intake on male sexual pheromone expression in the cockroach, *Nauphoeta cinerea*, and the implications this has for the operation of sexual selection. Sexual selection is well characterized in *N. cinerea*, occurring contemporaneously through male–male competition and female mate choice (Moore and Moore 1999). Both processes are mediated by a single sex-limited pheromone composed of three structural components: 2-methylthiazolidine (2MET), 4-ethyl-2-methoxyphenol (4E2M), and 3-hydroxy-2-butanone (3H2B) (Moore and Moore 1999). Females prefer males with higher levels of all three pheromone components (Moore and Moore 1999), whereas a higher level of 3H2B is known to increase the likelihood of a male being subordinate (Moore et al. 1997). Consequently, the pheromone compositions conferring male attractiveness to females and dominance status are opposing in this species (Moore and Moore 1999).

In our first experiment, we varied the overall nutrient concentrations (four levels) on each of six different protein:carbohydrate (P:C) ratios (24 unique diets, Table 1). This created a nutritional landscape with six nutritional rails along which male cockroaches could vary their protein and carbohydrate intake by eating more or less of a given diet (Fig. 1). We then measured the intake of protein and carbohydrates by males during sexual maturation and examined how this influenced sex pheromone expression (our trait of interest), as well as attractiveness and dominance (our reproductive fitness proxies). We also examined these nutrient effects on the accumulation of lipids and weight gain during sexual maturation, two measures commonly used as proxies of condition in empirical studies (Cotton et al. 2004; Tomkins et al. 2004). In our second experiment, we examined whether males prefer to consume protein or carbohydrates when provided with a choice of diets. Males were given the choice between diets that differed in both the P:C ratio and total nutrient concentration in four diet pairings (Table 1; Fig. 1). The amount of each diet consumed, and the corresponding intake of proteins and carbohydrates, was again measured during sexual maturation.

Table 1. **Protein and carbohydrate composition of the 24 artificial diets used in our feeding experiments. The total nutrients present in each diet is given as the sum of the percentage protein and percentage carbohydrate, with the remaining percentage consisting of indigestible crystalline cellulose. The four diets that were used in Experiment 2 are highlighted with bold text.**

Percentage Composition				
Protein (P)	Carbohydrate (C)	P+C	P:C ratio	Diet number
10	2	12	5:1	1
30	6	36	5:1	2
50	10	60	5:1	3
70	14	84	5:1	4
9	3	12	3:1	5
27	9	36	3:1	6
45	15	30	3:1	7
63	21	84	3:1	8
6	6	12	1:1	9
18	**18**	**36**	**1:1**	**10**
30	30	60	1:1	11
42	**42**	**84**	**1:1**	**12**
3	9	12	1:3	13
9	27	36	1:3	14
15	45	60	1:3	15
21	63	84	1:3	16
2	10	12	1:5	17
6	30	36	1:5	18
10	50	60	1:5	19
14	70	84	1:5	20
1.33	10.66	12	1:8	21
4	**32**	**36**	**1:8**	**22**
6.66	53.33	60	1:8	23
9.33	**74.66**	**84**	**1:8**	**24**

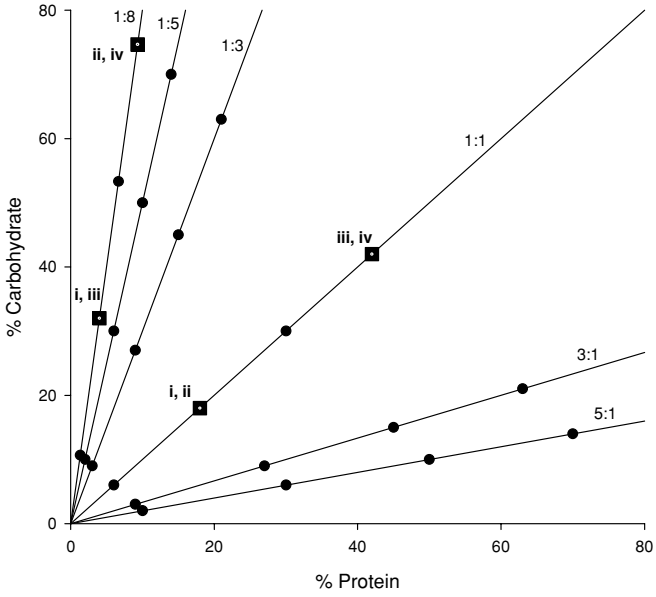

Figure 1. **The placement of the 24 diets used in Experiment 1 on the nutritional landscape. Dots represent the individual diets. The diet pairings used in our choice experiment (Experiment 2) are shown by the black squares and each roman numeral represents one of the four diet pairs. Lines represent the nutritional rails, labeled with the ratio of protein to carbohydrate that each rail is located on.**

Methods

EXPERIMENTAL ANIMALS

Individuals were obtained from our mass colony of *N. cinerea*, which consists of over 200,000 cockroaches of mixed sex dispersed between 10 large plastic containers (50 × 35 × 30 cm) that are maintained in three incubators set to 28 ± 1°C and a 14L:10D lighting regime. Each mass culture was fed weekly with a cup of dry rat chow and provided with water in two large test tubes (15-cm long, 3-cm diameter) plugged with cotton fiber. Mass cultures were cleaned every two months, at which point several thousand individuals were transferred between individual cultures to enforce gene flow. Substantial levels of genetic variation are known to exist in these mass cultures, with no evidence of inbreeding (Corley et al. 2001).

We established single-sex cultures of final instar nymphs that were isolated from our mass colony. Newly eclosed virgin adults were collected daily and randomly allocated to either a diet treatment (experimental males) or to be used in behavioral assays of male attractiveness and dominance in Experiment 1. Experimental males were housed individually in a plastic container (17 × 12 × 6 cm) and were provided their appropriate diet and water ad libitum. Virgin females and socially naïve, opponent males for behavioral assays were housed in plastic boxes (11 × 11 × 3 cm) with an ad libitum supply of rat chow and water. All individuals were only used once throughout both experiments, thus ensuring that the responses of the variables examined were not confounded by an individual's prior experience. All experimental animals were maintained in a large constant temperature room set at 28 ± 1°C with a 14L:10D lighting regime (i.e., identical conditions to the mass cultures).

ARTIFICIAL DIETS AND MEASURING FOOD INTAKE

We made artificial, dry, granular foods that varied in protein and carbohydrate content, based on the established protocol outlined in Simpson and Abisgold (1985). Proteins consisted of a 3:1:1 mixture of casein, albumen, and peptone and digestible carbohydrates were a 1:1 mixture of sucrose and dextrin. All diets contained Wesson's salts (2.5%), ascorbic acid (0.28%), cholesterol (0.55%), and vitamin mix (0.18%). After the appropriate dry weight of protein and carbohydrate had been added to the mixture, the remainder of the mixture was made up to the appropriate dilution with crystalline cellulose. Crystalline cellulose

is indigestible to most insects, however there is evidence that *N. cinerea* may have the ability to digest cellulose as the digestive enzyme cellulase has been found in their salivary glands (Wharton and Wharton 1965). Nonetheless, the contribution of cellulose digestion to overall carbohydrate intake is likely to be minimal, especially in relation to the intake of other carbohydrates shown in this study, as previous studies in the cellulase-producing cockroach *Blatella germanica* found cellulose digestion to be absent (Raubenheimer and Jones 2006) or, at most, minimal (Jones and Raubenheimer 2001). The diets used in each experiment are presented in Table 1 and Figure 1 shows the distribution of these diet treatments in nutritional space.

Each experimental male was given either one (no-choice trials in Experiment 1) or two (choice trials in Experiment 2) containers of food of measured dry weight on their first day of adulthood and food was changed every two days thereafter until 10 days posteclosion when males are sexually mature (Roth 1967). Food and water were provided in feeding platforms created by gluing the upturned plastic lid of vial (1.6-cm diameter, 1.6-cm deep) in the center of a plastic petri dish (5.5-cm diameter). The materials and design of the feeding platforms ensured that experimental males could not consume anything other than the artificial diet(s) and water and that food could be collected (in the petri dish) if spilled during feeding. Food was kept in a drying oven at 30°C for 48 h to remove moisture prior to weighing. Prior to weighing, any dried faeces were removed from the feeding platform using a pair of fine forceps.

Diet consumption was calculated as the difference in dry weight before and after feeding and converted to a weight of protein and carbohydrates ingested (e.g., 5 mg of 15P:45C ingested equals 0.75 mg of protein and 2.25 mg of carbohydrates). The total nutrient content of each diet is the percentage protein plus the percentage carbohydrates, with the remainder being the indigestible crystalline cellulose and micronutrients (e.g., the 15P:45C diet has a total nutrient content of 60%).

EXPERIMENT 1: NO CHOICE OF DIET ON SIX NUTRITIONAL RAILS AT FOUR CONCENTRATIONS

Measuring weight gain and lipid reserves

On the day of eclosion to adulthood (day 0), 10 replicate males were fed each of the 24 diets for 10 days ($n = 240$ cockroaches). Each experimental male was weighed at eclosion and thereafter every 2 days before feeding using an Ohaus electronic balance (Explorer® Pro). At 10 days posteclosion, experimental males were weighed and then frozen at −80°C. After thawing, males were prepared for lipid extraction by a single cut along the ventral side of their abdomen. Males were then dried at 60°C for 24 h after which their dry weight was measured. They were then placed in a test tube containing 20 mL of a 2:1 solution of dichloromethane: methanol and agitated for 48 h to extract lipids (Folch et al. 1957; Fischer 2006). Males were then removed from the solution and dried at 60°C for 24 h so that their dry weight after extraction could be measured. We measured the dry weight of cockroaches using a CAHN 28 microbalance (range: 1000 mg to 10 μg). We used the difference between the pre- and postextraction dry weights, expressed as a percentage of the initial preextraction dry weight, as our measure of lipid reserves. We used the slope of the linear regression of male weight across time as our measure of weight gain. It is important to note, however, that the analysis of absolute weight gain (i.e., difference in weights between day 0 and 10) gave identical results (Table 2). One male escaped during feeding. Thus, we had a total of 239 experimental males available for our final analysis of weight gain and lipid reserves.

Measuring sex pheromones

At 10 days posteclosion, 10 replicate males fed each of the 24 diets ($n = 240$ cockroaches) were frozen at −80°C. After freezing, the sternum was dissected from each male and blotted on tissue paper to remove any fat. The sternum was then completely submerged in 400 μL of HPLC grade dichloromethane containing an internal standard ([E,Z]-4–7-tridecadienyl acetate) at 10 ng/μl in a 1-mL conical vial, and allowed to soak at room temperature for 2 h, after which point the sternum was removed.

Using an autosampler (Agilent 7683B), 2 μL of the sample was injected into a DB-Wax column (30 m × 0.25 mm × 0.25 μm film thickness) housed in an Agilent 7890 GC coupled with an Agilent 5975 mass spectrometer, using helium as a carrier gas. The inlet temperature was set at 200°C and the injection was in pulsed split less mode. Following injection, the column was held at 50°C for 1.5 min before the temperature was raised at a rate of 10°C/min to 250°C, with a final hold time of 2 min. The MS transfer line was set at 240°C.

The mass spectrometer was operated in selected ion mode to limit the output to the target compounds (2-methylthiazolidine (2MET), 4-ethyl-2-methoxyphenol (4E2M) and 3-hydroxy-2-butanone (3H2B)), with analysis limited to the following ions: 45, 88, 103, 79, 107, 137, and 152. Samples were quantified against a multilevel calibration curve made using solutions containing the three target compounds at known concentrations. Analytical grade (99% pure) 3H2B was purchased from Sigma-Aldrich (St. Louis, MO), 4E2M was purchased from Pfaltz and Bauer (Waterbury, CT), and 2MT was purchased from Endeavor Specialty Chemicals (Daventry, UK). One sample returned uncharacteristically low levels of pheromones and was excluded from the analysis. Thus, there were a total of 239 experimental males available for the final analysis.

Measuring male attractiveness

At 10 days posteclosion, 15 replicate experimental males fed each of the 24 diets ($n = 360$ cockroaches) were randomly assigned

Table 2. The linear and nonlinear effects of protein and carbohydrates intake on male sex pheromones, attractiveness, dominance, and two commonly used proxies of condition (weight gain and lipid reserves).

	Linear effects		Nonlinear effects		
Response variable	Protein (P)	Carbohydrates (C)	P×P	C×C	P×C
3-hydroxy-2-butanone					
Slope±SE	0.001±0.010	0.035±0.005	$3.3\times10^{-5}\pm0.000$	$-1.3\times10^{-4}\pm0.000$	$-3.4\times10^{-4}\pm0.000$
t_{234}	0.113	7.527	0.218	4.207	2.637
P	0.910	0.0001	0.827	0.0001	0.009
4-ethyl-2-methoxyphenol					
Slope±SE	0.026±0.014	0.024±0.005	$-6.5\times10^{-5}\pm0.000$	$-6.0\times10^{-5}\pm0.000$	$-4.2\times10^{-4}\pm0.000$
t_{234}	1.556	5.031	0.414	1.924	3.128
P	0.121	0.0001	0.680	0.056	0.002
2-methylthiazolidine					
Slope±SE	0.014±0.011	0.018±0.005	$1.7\times10^{-4}\pm0.000$	$-3.6\times10^{-5}\pm0.000$	$-2.1\times10^{-4}\pm0.000$
t_{234}	1.225	3.501	1.017	1.098	1.504
P	0.222	0.001	0.310	0.273	0.134
Attractiveness					
Slope±SE	0.007±0.009	0.023±0.007	0.002±0.139	−0.035±0.045	−0.377±0.203
t_{349}	0.707	3.171	0.016	0.783	1.861
P	0.480	0.002	0.987	0.434	0.064
Dominance index					
Slope±SE	0.015±0.010	−0.001±0.004	$-2.2\times10^{-4}\pm0.000$	$4.1\times10^{-6}\pm0.000$	$-1.5\times10^{-5}\pm0.000$
t_{348}	1.568	0.150	1.466	0.127	0.092
P	0.118	0.881	0.144	0.899	0.927
Absolute dominance					
Slope±SE	0.008±0.005	0.001±0.002	−0.099±0.074	−0.005±0.016	−0.044±0.081
t_{348}	1.61	0.68	1.34	0.33	0.54
$\beta_{rand} \geq \beta_{real}$	515	2455	9090	6295	7090
P	0.10	0.49	0.18	0.74	0.58
Weight gain					
Slope±SE	0.023±0.011	0.022±0.005	$-5.0\times10^{-5}\pm0.000$	$-9.1\times10^{-5}\pm0.000$	$4.2\times10^{-5}\pm0.000$
t_{234}	2.106	4.556	0.258	1.889	0.254
P	0.036	0.0001	0.796	0.060	0.800
Absolute weight gain					
Slope±SE	0.001±0.000	0.001±0.000	−0.001±0.007	−0.003±0.002	0.001±0.006
t_{234}	2.103	4.801	0.197	1.805	0.197
P	0.036	0.0001	0.844	0.072	0.844
Lipid reserves					
Slope±SE	−0.011±0.012	0.010±0.004	$-1.7\times10^{-4}\pm0.000$	$4.9\times10^{-6}\pm0.000$	$-2.4\times10^{-5}\pm0.000$
t_{234}	0.878	2.242	0.774	0.090	0.133
P	0.381	0.026	0.439	0.928	0.894

Response variables were *Z*-transformed prior to analysis to ensure differences in scale did not alter the magnitude of the relationship with nutrients. Although the sign of the linear slope describes the direction of the relationship between nutrients and the response variable, the nonlinear slope describes the curvature of this relationship, with a negative slope indicating a convex relationship (i.e., a peak on the landscape) and a positive slope indicating a concave relationship (i.e., a trough on the landscape).

a 10-day-old, virgin female. The experimental male was then introduced into a plastic container (17 × 12 × 6 cm) with greased sides to prevent escape and given 5 min to acclimate. The female was then added to the container and the courtship and mating behavior of the experimental male recorded for 10 min under red light. Courtship interactions in *N. cinerea* involve a complex and stereotypical sequence of behaviors (Roth 1964; Moore and Breed 1986). Once the male has initiated antennal contact with the female, he presents his posterior end to the female and raises his wings to expose his sternal glands located on the dorsal surface of his abdomen. After a short period of assessment, the female either decides to mount the male and initiate mating or rejects his mating

attempts. If she mounts, the male inserts his phallomere under the subgenital plate and the pair rotates 180°, where they remain motionless in copula for approximately 12 min until mating is terminated.

We measured male attractiveness as the interval between the male performing a wing raise and gaining a successful mounting (i.e., a mounting that resulted in a successful mating) by the female. This time interval is therefore a measure of courtship speed, with a smaller interval meaning that the male is more attractive to the female (Moore and Breed 1986; Moore and Moore 1999). To make male attractiveness directly comparable to our other response variables, we used the inverse of this measure in our analysis (i.e., values closer to 0 mean the male is more attractive). It is important to note that a previous experiment on *N. cinerea* has shown that this short range measure of attractiveness correlates strongly with the longer range attractiveness of males determined in an olfactometer without direct contact between the male and female (Moore and Moore 1999). Six trials did not result in a successful mating and were excluded from our analysis. Thus, we had a total of 354 experimental males for our final analysis.

Measuring male dominance

At 10 days posteclosion, 15 replicate experimental males fed each of the 24 diets ($n = 360$ cockroaches) were randomly assigned one of the 10-day-old, socially naïve male competitors who had been housed individually since the day of eclosion. One male in the pair, chosen at random, was marked with a disc of filter paper glued to the pronotum for identification and the experimental male was then introduced into a plastic container ($17 \times 12 \times 6$ cm) with greased sides to prevent escape. After 5 min of acclimation, the random male competitor was introduced into the container. The behavior of these males was observed continuously under red light for 10 min and we recorded the outcome of the contest, as well as the time taken to reach this outcome. Aggressive behaviors that are characteristic of a dominant male include lunging, butting, biting, grappling, stilt-walking, and patrolling a territory and the subordinate male typically responds by retreating and/or adopting a crouching position (Bell and Gorton 1978; Moore et al. 1995). Behavioral trials where the outcome was unclear after 10 min of observation ($n = 7$ pairs) were not included in the final analysis. Thus, a total of 353 experimental males were available for the final analysis.

We scored the dominance status of the experimental male in each pair as 1 or −1, depending on whether he was dominant or subordinate to his competitor, respectively. We then calculated a weighted dominance index by multiplying each experimental male's absolute dominance score by the following weighting factor:

$$\text{weighting} = \frac{1}{\sqrt{t}} - \frac{1}{\sqrt{600}},$$

where t is the time taken for the outcome to be reached. This weighted dominance index was normally distributed. If the experimental male gains dominance quickly, this gives the highest possible weighted dominance score (1). Conversely, if he becomes subordinate very quickly, this gives the lowest possible score (−1). If the experimental male takes a long time to become either dominant or subordinate, this gives a score close to 0.

It is important to note that the linear and nonlinear effects of protein and carbohydrate consumption were identical irrespective of whether we analyzed absolute dominance or this weighted dominance score (Table 2). Because absolute dominance is a binary measure and not normally distributed we tested the significance of terms in the model using a randomization test implemented in Poptools (version 3). We started by first running the full parametric model including all linear and nonlinear terms. We then randomly shuffled absolute dominance scores across diets to obtain an expected distribution under random association and used a Monte-Carlo analysis to simulate this 10,000 times. The number of permutations (out of 9,999) in which the gradient pseudo-estimate was greater than or equal to the original estimate was calculated and this proportion converted to a two-tailed probability value following the protocol outlined in Manly (1997).

Statistical analyses

We used a multivariate response-surface approach (Lande and Arnold 1983) to estimate and visualize the linear and nonlinear effects of protein and carbohydrate intake on our response variables (pheromones, attractiveness, dominance, weight gain and percentage lipids). Response surfaces were fitted in SPSS (version 15) and visualized using nonparametric thin-plate splines in R (version 2.9.1).

We used a sequential model building approach to assess whether the linear and nonlinear effects of protein and carbohydrate ingestion differed for our response variables (Draper and John 1988; Chenoweth and Blows 2005). As our different responses variables were measured in different units, it was necessary to standardize them for a statistical comparison. Prior to comparison, we therefore standardized each response variable to a mean of zero and standard deviation of one using a *Z*-transformation. We then included a dummy variable, response type (*RT*), in a reduced model containing only the standardized linear terms

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^{n} \beta_i N_i + \varepsilon, \qquad (1)$$

where R is our standardized response measure, N_i refers to the intake of the ith nutrient, n represents the number of nutrients contained in the model, and ε is the unexplained error. From (1), the unexplained (i.e., residual) sums of squares for this reduced model (SS_r) were compared to the same quantity (SS_c) from a

second (complete) model that included all of the terms in (1) with the addition of the terms $\alpha_i N_i RT$, which represents the linear interaction of RT and the ith nutrient:

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^{n} \beta_i N_i + \sum_{i=1}^{n} \alpha_i N_i RT + \varepsilon. \quad (2)$$

A partial F-test (Bowerman and O'Connell 1990) was used to compare SS_r and SS_c from (eq. 1) and (eq. 2), respectively

$$F_{a,b} = \frac{(SS_r - SS_c)/a}{SS_c/b}, \quad (3)$$

where a is the number of terms that differ between the reduced and complete model and b is the error degrees of freedom for SS_c.

To test whether the quadratic effect of nutrient intake differed between response variables, the SS_r from the reduced model

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^{n} \beta_i N_i + \sum_{i=1}^{n} \alpha_i N_i RT + \sum_{i=1}^{n} \beta_i N_i^2 + \varepsilon \quad (4)$$

was compared to the SS_c of the complete model

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^{n} \beta_i N_i + \sum_{i=1}^{n} \alpha_i N_i RT + \sum_{i=1}^{n} \beta_i N_i^2 + \sum_{i=1}^{n} \alpha_i N_i^2 RT + \varepsilon \quad (5)$$

using equation (3).

To test whether correlational effects of nutrient intake on response variables differed, the SS_r from the reduced model

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^{n} \beta_i N_i + \sum_{i=1}^{n} \alpha_i N_i RT + \sum_{i=1}^{n} \beta_i N_i^2 + \sum_{i=1}^{n} \alpha_i N_i^2 RT + \sum_{i=1}^{n} \sum_{j \geq 1}^{n} \beta_{i_j} N_i N_j + \varepsilon \quad (6)$$

was compared to the SS_c of the complete model

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^{n} \beta_i N_i + \sum_{i=1}^{n} \alpha_i N_i RT + \sum_{i=1}^{n} \beta_i N_i^2 + \sum_{i=1}^{n} \alpha_i N_i^2 RT + \sum_{i=1}^{n} \sum_{j \geq 1}^{n} \beta_{i_j} N_i N_j + \sum_{i=1}^{n} \sum_{j \geq 1}^{n} \alpha_{i_j} N_i N_j RT + \varepsilon \quad (7)$$

using equation (3).

In summary, the comparison of model (eq. 1) versus (eq. 2), (eq. 4) versus (eq. 5), and (eq. 6) versus (eq. 7) provides a test for the overall significance of the interaction between response type and the linear, quadratic, and correlational effects of nutrient intake, respectively. Therefore, significant differences in these model comparisons, as detected with a partial F-test, demonstrates that the linear, quadratic, and/or correlational effects of nutrient intake on the response variables differ, respectively. We also inspected the interaction of individual nutrients with the response variable terms from the full model (eq. 7) to determine which of the nutrients were responsible for the significance of the overall partial F-test.

EXPERIMENT 2: MEASURING DIET INTAKE UNDER CHOICE

Thirty males were assigned at random to each of the four possible diet pairings that differ in both the ratio of protein to carbohydrates, as well as total nutrients (P:C(total nutrient): Pair 1: 1:1(36) versus 1:8(36), Pair 2: 1:1(36) versus 1:8(84), Pair 3: 1:1(84) versus 1:8(36), and Pair 4: 1:1(84) versus 1:8(84). This corresponds to diets 10, 12, 22, and 24 in Table 1 and provides good coverage of carbohydrate space on the nutritional landscape (Fig. 1). As outlined above, the consumption of both diets was measured every two days for 10 days posteclosion.

For each diet pair, we calculated the intake of protein and carbohydrates that would be expected if experimental males consumed each of the diets at random. This expected value was calculated by (1) halving the total amount of both diets consumed, (2) converting this to an expected weight of protein and carbohydrate ingested for each diet by multiplying by the nutrient concentration of the diet, and (3) summing the total amount of expected protein and carbohydrates ingested on both diets. For example, if a male cockroach consumed 10 mg of diet A (18:18(36)) and 20 mg of diet B (4:32(36)), the expected consumption of both diets is 15 mg and this equals 2.7 mg each of protein and carbohydrates on diet A and 0.6 mg of protein and 4.8 mg of carbohydrates on diet B. The total amount of protein ingested if males consumed diets at random is therefore 3.3 mg and the total expected amount of carbohydrates is 7.5 mg.

Statistical analyses

To determine whether male cockroaches preferentially consumed one of the diets over the other in each diet pairing, we compared the absolute consumption of each diet using a paired t-test. However, this does not account for the fact that the diets have different concentrations of protein and carbohydrates so that a male may actually eat more of a less concentrated diet to increase protein and/or carbohydrate intake (i.e., compensatory feeding).

Consequently, we directly compared the observed total protein and carbohydrates ingested in each diet pairing by subtracting the expected ingestion of these nutrients from the observed values and comparing this difference to a mean of zero (i.e., if cockroaches were eating at random) using a one-sample t-test. A value greater than zero therefore means that a male has consumed more protein or carbohydrates than expected, a value less than zero means he has consumed less than expected, whereas a

value that does not differ significantly from zero means that the male has consumed protein and carbohydrates equally from both diets.

The total intake of protein and carbohydrates was compared across diet pairs with a multivariate analysis of variance (MANOVA) and pairwise Tukey's honestly significantly different (HSD) contrasts. The intake target ratio of nutrients (Simpson et al. 2004) was calculated as the combined mean protein and carbohydrate intake across these four diet pairings.

Results

EXPERIMENT 1: NO CHOICE OF DIET ON SIX NUTRITIONAL RAILS AT FOUR CONCENTRATIONS

The nutritional landscapes obtained from our first experiment (Fig. 2A–C) clearly show that the expression of all three components of the male sexual pheromone was significantly influenced by the intake of carbohydrates, whereas the intake of protein had a relatively minor effect (Table 2). Both 3H2B and 4E2M were characterized by a major peak of expression maximized on diets containing a low P:C ratio and a secondary minor peak at intermediate levels of carbohydrate intake and medium-to-high protein intake (Fig. 2A,B, Table 2). In contrast, the expression of 2MT increased linearly with carbohydrate intake and was not influenced by protein intake (Fig. 2C, Table 2). The only statistically significant difference between these nutritional landscapes was in the linear effect of nutrient intake on pheromone expression (linear: $F_{2,708} = 9.18$, $P = 0.0001$; quadratic: $F_{2,702} = 2.60$, $P = 0.08$; correlational: $F_{1,699} = 1.26$, $P = 0.26$), resulting primarily from differences in how responsive each pheromone component was to carbohydrate intake (protein: $F_{2,699} = 1.36$, $P = 0.26$; carbohydrates: $F_{2,699} = 3.18$, $P = 0.04$). On average, 3H2B increased more per unit of carbohydrate ingested than did 4E2M and 2MT (Table 2).

Male attractiveness increased linearly with carbohydrate intake but was not influenced by protein intake (Table 2). The nutritional landscape for male attractiveness (Fig. 2D) shows a high degree of similarity with those for male pheromone expression (Fig. 2A–C). Indeed, a formal comparison of these nutrient landscapes reveals that they do not differ significantly (linear: $F_{2,1059} = 2.35$, $P = 0.10$; quadratic: $F_{2,1051} = 2.87$, $P = 0.06$; correlational: $F_{1,1047} = 1.23$, $P = 0.27$). Thus, consuming a high carbohydrate diet increases the expression of all three components of the sex pheromone and this makes a male more attractive to females. In contrast, the intake of either nutrient did not significantly influence male dominance (Table 2). This was irrespective of whether we analyzed absolute dominance scores or our index of dominance (Table 2).

We found that nutrient intake had different effects on lipid accumulation and weight gain (Table 2). As shown for the sex pheromone and male attractiveness, lipid accumulation increased linearly with carbohydrate intake but was not influenced by the intake of protein (Table 2, Fig. 2E). In contrast, weight gain increased linearly with the intake of protein and carbohydrates (Table 2, Fig. 2F). Importantly, the nutritional landscapes for both commonly used proxies of condition differed significantly from the landscape for male attractiveness in their linear effects of nutrient intake (lipid accumulation, linear: $F_{2,587} = 8.42$, $P = 0.0001$; quadratic: $F_{2,583} = 0.53$, $P = 0.59$; correlational: $F_{1,581} = 1.61$, $P = 0.21$; weight gain, linear: $F_{2,587} = 16.67$, $P = 0.0001$, quadratic: $F_{2,583} = 0.09$, $P = 0.91$; correlational: $F_{1,581} = 2.39$, $P = 0.12$). This resulted from lipid accumulation being less responsive to carbohydrate intake than male attractiveness (protein: $F_{1,581} = 1.10$, $P = 0.27$; carbohydrates: $F_{1,581} = 4.09$, $P = 0.04$) and protein intake influencing weight gain but not attractiveness (protein: $F_{1,581} = 5.23$, $P = 0.02$; carbohydrates: $F_{1,581} = 0.57$, $P = 0.45$) (Table 2).

EXPERIMENT 2: MEASURING DIET INTAKE UNDER CHOICE

For each diet pair, males consumed significantly more of the diet containing more carbohydrates than more protein, irrespective of total nutrient content (Pair 1: $t_{29} = 6.27$, $P = 0.0001$; Pair 2: $t_{29} = 17.26$, $P = 0.0001$; Pair 3: $t_{29} = 17.36$, $P = 0.0001$; Pair 4: $t_{29} = 5.40$, $P = 0.0001$). The actual P:C ratio of alternate diets in each pair are provided above each bar and the total nutrient content of each diet provided within the bar in bold (Fig. 3A). Not surprisingly, this resulted in a significantly higher intake of carbohydrates than expected if they fed indiscriminately (Pair 1: $t_{29} = 9.41$, $P = 0.0001$; Pair 2: $t_{29} = 17.64$, $P = 0.0001$; Pair 3: $t_{29} = 20.44$, $P = 0.0001$; Pair 4: $t_{29} = 13.01$, $P = 0.0001$) and, with the exception of diet pair 3, consumed significantly less protein than expected (Pair 1: $t_{29} = 6.23$, $P = 0.0001$; Pair 2: $t_{29} = 17.26$, $P = 0.0001$; Pair 4: $t_{29} = 5.40$, $P = 0.0001$). In diet pair 3, the preference for the diet with more carbohydrates meant that males necessarily consumed significantly more protein than expected if they fed indiscriminately (Pair 3: $t_{29} = 17.36$, $P = 0.0001$) (Fig. 3B). Correspondingly, the intake of protein and carbohydrates by males differed significantly across our four diet pairs (MANOVA: Pillai's trace = 1.41, df = 6,232, $P = 0.0001$; all pairwise contrasts $P < 0.05$) (Fig. 4). Thus, although male *N. cinerea* do not feed indiscriminately, nutrient intake is not as tightly regulated as in some insect species (e.g., *Spodoptera littoralis*, Simpson et al. 2004), being insufficient to fully compensate for the reduced levels of carbohydrates contained in diet pairs 1 and 3. The mean intake point (intake target) was 1:3.2 (P:C). Despite the apparent inability of males to tightly regulate nutrient intake, their intake target remained in close proximity to the regions of maximal expression of the male sex pheromones and attractiveness on the nutritional landscapes (Fig. 2).

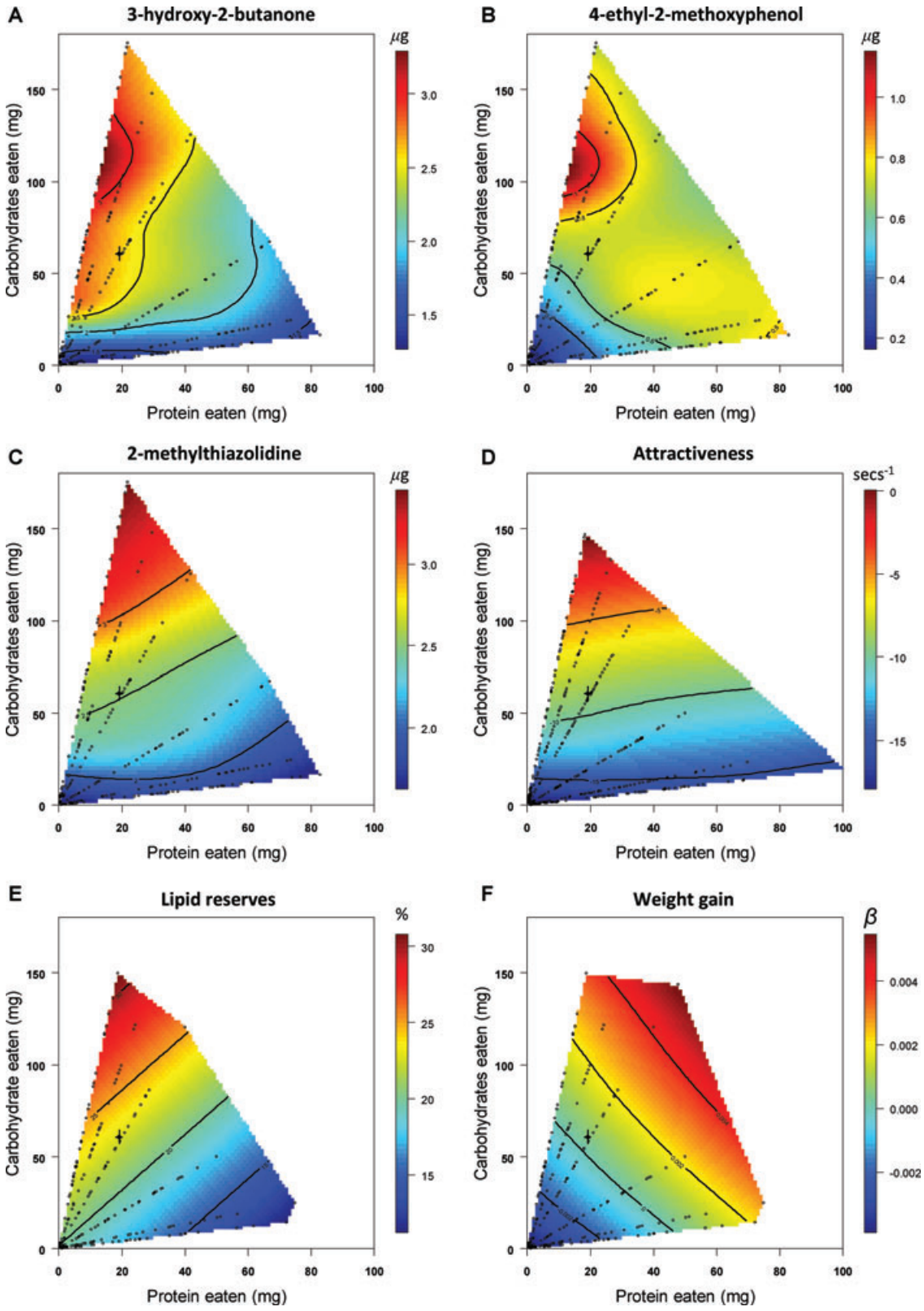

Figure 2. Nutritional landscapes illustrating the effects of protein and carbohydrate intake on the expression of the three male sex pheromones, (A) 3-hydroxy-2-butanone, (B) 4-ethyl-2-methoxyphenol, and (C) 2-methylthiazolidine, as well as (D) male attractiveness and two commonly used proxies of condition, (E) lipid reserves and (F) weight gain. High values are given in red and low values in blue and black dots are the intake data for individual cockroaches. The black cross on each landscape represents the regulated intake point (±SEs) presented in Figure 4.

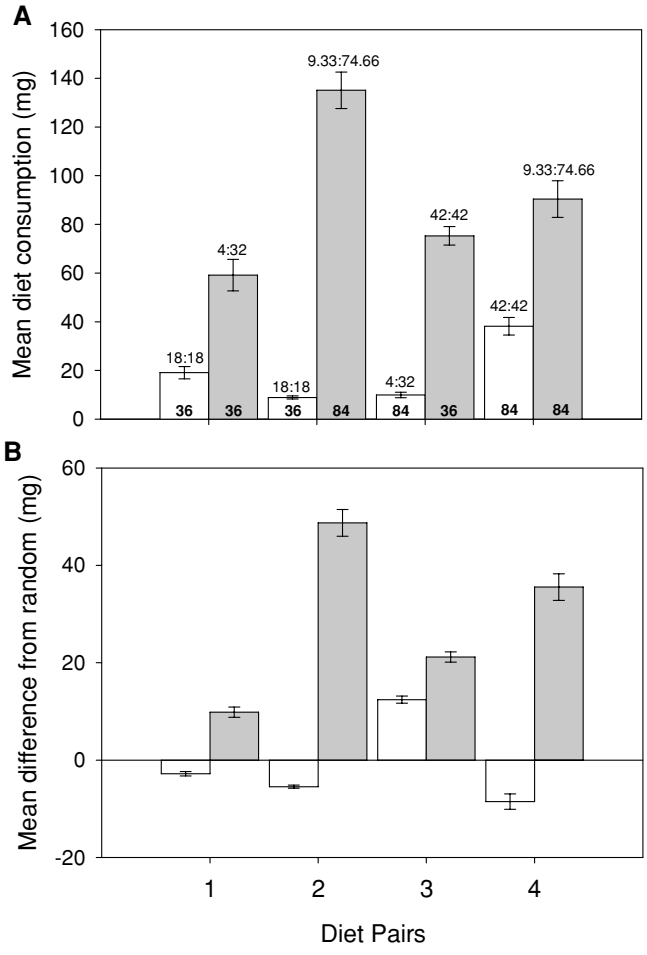

Figure 3. **The mean (±SE) amount of diet consumed and the associated intake of proteins and carbohydrates in each of the diet pairs. (A) The absolute consumption of each diet. Gray bars represent high carbohydrate diet, white bars the high protein diet. The actual P:C ratio of alternate diets in each pair are provided above each bar and the total nutrient content of each diet provided within the bar in bold. For each diet pair, males consumed significantly more of the diet containing more carbohydrates (gray) than more protein (white), irrespective of total nutrient content. (B) Difference in protein (white bars) and carbohydrates (gray bars) from consumption expected if males fed at random. For each diet pair, males consumed significantly more carbohydrates (gray) than expected if they fed indiscriminately and, with the exception of diet pair 3, consumed significantly less protein than expected (for details see Results- Experiment 2).**

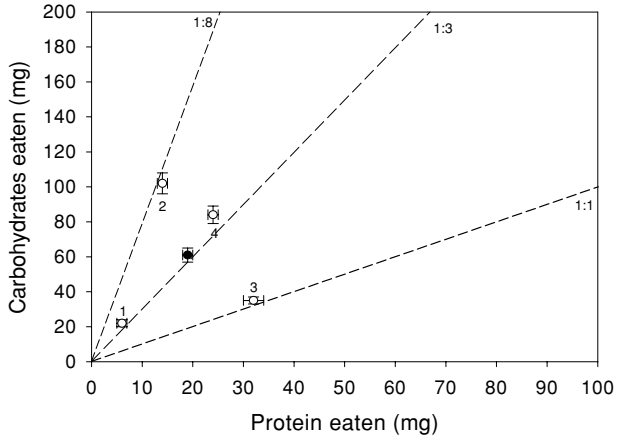

Figure 4. **The mean (±SE) points of protein (P) and carbohydrate (C) intake for the four diet pairs (open) and the regulated intake point (black), calculated as the mean P and C intake across diet pairs. The consumption of P and C differed significantly between the diets and the regulated intake point was estimated at 1:3.2 (for details see Results- Experiment 2). The dashed lines represent the expected intake of P and C if males regulated their intake at a P:C ratio of 1:8, 1:3 and 1:1 (left to right of figure), respectively.**

Discussion

Collectively, our results demonstrate that male sex pheromone expression in *N. cinerea* is dependent on carbohydrate intake but is largely unrelated to protein intake. Condition-dependent pheromone expression, in turn, increases a male's attractiveness but has little effect on his dominance in male–male competition. Dominant males are known to mate more often, but not exclusively, and attractiveness also plays a crucial role in determining male reproductive fitness (Moore and Moore 1999). Thus, carbohydrate intake plays an important role in determining total fitness in this species. Moreover, when given the opportunity to choose between diets, males actively choose the richer source of carbohydrates. The link we show here between nutrient intake, pheromone expression, and attractiveness largely agrees with previous studies on *N. cinerea*. Dietary restriction reduces pheromone expression, particularly levels of 3H2B, reducing a male's attractiveness to females (Clark et al. 1997). Furthermore, experimentally increasing all three components of the male sex pheromone independent of underlying condition increases male attractiveness but does not influence his social status (Moore et al. 1997). Here we have shown that carbohydrates play a large role in mediating this relationship and show that males actively regulate the intake of this important nutrient.

Our results have a number of implications for the study of condition dependence. First, our work shows that the acquisition of specific nutrients from the diet can be more important to the expression of sexual traits than the total nutrient intake. In *N. cinerea*, the expression of the male sex pheromone is highly dependent on carbohydrate intake but is largely independent of protein intake. Cockroaches, like the majority of insects, use carbohydrates as their main energy source, storing any excess as lipids for future metabolic use (Nation 2001). In at least one cockroach species, *B. germanica*, lipids are known to be the major precursor for the biosynthesis of sex pheromones (Chase et al. 1990, 1992; Schal et al. 1991). Thus, it is likely that the increase

in lipid reserves we observed with carbohydrate intake (Fig. 2E) mediates the increased expression of the sex pheromone in *N. cinerea*. Interestingly, in a study exploring the effects of protein and carbohydrate intake on ageing in crickets (*Teleogryllus commodus*), carbohydrate consumption by males was shown to increase lipid stores and the time spent calling (a sexually selected trait) (Maklakov et al. 2008). In this species, however, lipids are likely used as an energy-rich source (Nation 2001) to directly meet the high metabolic demands of calling (Kavanagh 1987). It is therefore possible that the positive relationship between carbohydrate intake and attractiveness in *N. cinerea* was not solely due to the increase in pheromone production, but also due to the increase in available energy, and that males with larger lipid reserves were also able to display more energetically to females. Given this possibility, it is surprising that we found no effect of carbohydrate intake on dominance during male–male competition as these contests are likely to be more energetically demanding.

Second, we show that whenever an organism can assess the nutritional quality of the food it consumes, condition is likely to be a dynamic process. Individuals can face extreme fitness costs of being in low condition through both decreased fecundity and survival, and over ingestion of certain nutrients can exacerbate these costs. For example, previous studies have found negative effects of proteins on longevity in cockroaches (Haydak 1953; Mullins and Cochran 1975). Therefore it is not surprising that there is strong selection for effective regulatory mechanisms that control the amount and balance of nutrients ingested (Raubenheimer and Simpson 2004). Indeed, the regulation of nutrient intake toward higher levels of carbohydrates appears to be a general feature in many animal species (Fernstrom 1983), including the cockroach species thus far examined (e.g., Cohen et al. 1987; Cohen 2001; Jones and Raubenheimer 2001). In at least one cockroach species, *Rhyparobia madera*, carbohydrate intake appears to be regulated by the neurotransmitter serotonin (Cohen 2001). Whether a similar mechanism exists to regulate protein intake is unknown. Work on the American cockroach, *Periplaneta americana*, suggests that odor-associated learning may also play an important role, at least in distinguishing between food items that are high in protein from those high in carbohydrates (Gadd and Raubenheimer 2000). Given the negative effects of proteins and the role of lipids as an energy source for not only mate attraction as discussed above but also a variety of other activities (e.g., during flight Beenakkers et al. 1984), the observed preference for carbohydrates is mostly likely also due to selection on a range of other fitness proxies affecting both fecundity and survival.

Finally, our work illustrates that proxies of condition commonly used in sexual selection studies are likely to differ greatly in their value. For a proxy to be meaningful it must reliably predict condition or, at a minimum, be influenced by resource acquisition in the same way as fitness. This was not the case for weight gain in *N. cinerea* which increased with both protein and carbohydrate intake (Table 2, Fig. 2F) and therefore only reflected the total amount of nutrients consumed. This contrasted with the effect of carbohydrate intake on our fitness proxy, male attractiveness. Lipid reserves provided a better condition proxy in *N. cinerea* but were still less responsive than male attractiveness to carbohydrate intake (Table 2, Fig. 2E). This may reflect the partitioning of dietary carbohydrates between storage as lipids and use as a direct energy source (Nation 2001).

It is likely that sexual traits in many species are influenced by the intake of specific nutrients rather than overall nutrient intake, as we have shown in this study. Further evidence for specific nutrient effects come from the distribution of carotenoids between the immune system, tissue repair and maintenance, and the bright displays of many birds and fish, and the resulting effects on their attractiveness to females (Olson and Owens 1998; Cotton et al. 2004). In such cases where specific nutrients are deposited directly in the sexual traits, it is clear that energy reserves are unlikely to reflect true condition (i.e., carotenoids ingested). Our study and that of Maklakov et al. (2008) show that there can be complex relationships between other types of sexually selected traits (in these studies pheromones and acoustic signaling, respectively) and specific nutrients. Nutritional geometry provides a powerful tool to explore the relationships between specific nutrients and sexual trait expression. However, manipulating diet and measuring intake is difficult in the field. One solution could be to bring individuals in to the laboratory to measure nutrient intake and its effects and determine suitable proxies of condition that can be measured in the field. Until such underlying relationships are known, we recommend caution be taken when interpreting proxies of condition in empirical studies (Cotton et al. 2004; Tomkins et al. 2004).

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