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Protein and carbohydrate intake influence sperm number and fertility in male cockroaches, but not sperm viability

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It is commonly assumed that because males produce many, tiny sperm, they are cheap to produce. Recent work, however, suggests that sperm production is not cost-free. If sperm are costly to produce, sperm number and/or viability should be influenced by diet, and this has been documented in numerous species. Yet few studies have examined the exact nutrients responsible for mediating these effects. Here, we quantify the effects of protein (P) and carbohydrate (C) intake on sperm number and viability in the cockroach *Nauphoeta cinerea*, as well as the consequences for male fertility. We found the intake of P and C influenced sperm number, being maximized at a high intake of diets with a P : C ratio of 1 : 2, but not sperm viability. The nutritional landscapes for male fertility and sperm number were closely aligned, suggesting that sperm number is the major determinant of male fertility in *N. cinerea*. Under dietary choice, males regulate nutrient intake at a P : C ratio of 1 : 4.95, which is midway between the ratios needed to maximize sperm production and pre-copulatory attractiveness in this species. This raises the possibility that males regulate nutrient intake to balance the trade-off between pre- and post-copulatory traits in this species.

1. Introduction

The production of large and small gametes, known as anisogamy, is a widespread phenomenon in the animal kingdom [1,2]. Historically, evolutionary biologists have used this asymmetry in gamete size between the sexes to explain the origin of sex differences in parental investment strategies [3,4] and sexual conflict [5]. These arguments are based on the assumption that because male gametes (sperm) are typically small and numerous compared with female gametes (ova), they should be cheap to produce [3–5]. There are, however, a growing number of studies challenging this assumption and showing that sperm production often comes at a substantial cost to males [6,7], although it is unlikely that these costs will ever exceed those experienced by females when producing ova [8].

Several lines of empirical evidence suggest that sperm are costly to produce. First, males in a wide range of species have been shown to strategically tailor the number of sperm in their ejaculate in response to a variety of stimuli, including the perceived risk of sperm competition [9], female mating status [10] and female quality [11], as well as in response to the quality of environment for developing offspring [12]. Moreover, there is a growing number of studies showing that males can also adjust the proportion of viable sperm in their ejaculate to the risk of sperm competition [13] and female mating status [14]. Second, males frequently show a significant reduction in sperm number after multiple ejaculations, indicative of sperm depletion [15]. For example, sperm number is reduced by 65% in large male sea slugs (*Chelidonura sandrana*) across four successive matings [15]. Third, sperm production has been

associated with an elevated metabolic rate within [16] and across [8] species. In some species, this elevation in metabolic rate is likely to represent a significant cost to the daily energy budget. For example, in Japanese macaques (*Macaca fuscata*), it is estimated that males use between 0.8% and 6.0% of their daily basal metabolic rate for ejaculate production during the breeding season [16]. Finally, sperm number is traded against sperm size across species [17], and against a range of different traits within species (i.e. lifespan [18], body weight [19], immune function [20], growth [21], mate guarding behaviour [22]). Furthermore, sperm viability is also traded against attractiveness [23] and immune function [24] in several species.

If sperm are indeed costly, diet should have an effect on the number and/or the viability of sperm produced. Diet is known to influence sperm production in numerous mammalian species [25], including humans [26]. There are, however, few studies that have examined the effects of diet on sperm production in insects. Moreover, the small number of studies that have examined this relationship in insects have yielded equivocal results. Queensland fruitflies (*Bactrocera tryoni*) [27], *Drosophila melanogaster* [28], red flour beetles (*Tribolium castaneum*) [29] and Indian meal moth (*Plodia interpunctella*) [30] all show an increase in sperm production on higher-quality diets, whereas studies on ladybirds (*Adalia bipunctata*) [31] and Mediterranean fruitflies (*Ceratitis capitata*) [32] show the reverse pattern. Only two of these studies have examined the consequences of diet-induced changes in sperm production for male fitness: the increase in sperm production observed on a high-quality diet results in a higher paternity in *T. castaneum* [29] but not in *D. melanogaster* [28]. To date, only a single study on honeybees (*Apis mellifera*) has examined the effect of diet on sperm viability, showing that pollen restriction has little effect on sperm viability [33].

The ovoviviparous cockroach *Nauphoeta cinerea* has been a useful model for studying post-copulatory sexual selection [34–39]. Male *N. cinerea* produce a complex ejaculate (the spermatophore) consisting of sperm enclosed in a membranous sac (the ampulla) that is embedded in a gelatinous, proteinaceous mass (the spermatophylax) [40]. If the spermatophylax is above a threshold size, it stimulates a stretch receptor in the female bursa copulatrix that inhibits remating behaviour [35,41,42]. Most males are able to produce a spermatophylax that exceeds this threshold and therefore typically avoid sperm competition when mating with a virgin female [42]. Sperm competition is not completely eliminated by this mechanism, however, as females can remate after the parturition of their first clutch [40,41] and can store sperm for their entire lifespan [41,42]. There is already compelling evidence that sperm production is costly in *N. cinerea*. Manipulation of the social environment during larval [36] and adult [38] stages has shown that males tailor their sperm number according to their perceived risk of sperm competition. Males also readily become sperm-depleted over consecutive matings, and this significantly reduces male fertility [35,39]. For example, ampulla size and sperm viability both decrease considerably across four consecutive matings (approx. 70% and 50%, respectively), and this results in a 35% decrease in male fertility [35]. Females are able to use cuticular hydrocarbons transferred during mating to discriminate against sperm-depleted males in mate choice [35,36], although their ability to assess these chemical cues becomes less effective as the time between successive matings increases [39]. Finally, there are negative genetic correlations between sperm number and most other components of the ejaculate, including spermatophore mass, ampulla mass, testis mass and sperm viability [34]. This suggests that sperm number is traded against these other ejaculate components and is unlikely to evolve independently [34]. We currently do not know whether diet influences sperm number and/or viability in *N. cinerea*.

A limitation of all previous studies examining the relationship between diet and sperm production in insects is that an explicit nutritional framework was not used to manipulate diet quality [27–32]. Thus, it is not possible to separate the effects of total nutritional content (i.e. calories) from the intake of specific nutrients in these studies. Here, we use the geometrical framework (GF) for nutrition, a multidimensional approach that enables the independent effects of calories on an individual's performance to be partitioned from the intake of specific nutrients [43], to determine the effects of protein (P) and carbohydrate (C) intake on sperm production and viability in *N. cinerea* and the resulting effects on male fertility. In total, we conducted three separate experiments. In our first experiment, we restricted male cockroaches to feed on one of 24 artificial, holidic diets during sexual maturation to quantify the effects of P and C intake on sperm number and sperm viability (experiment 1). Next, we restricted a second group of male cockroaches to feed on these diets during sexual maturation and then mate them to a virgin female. These females were then maintained on a standard diet until parturition and offspring counted to quantify the effects of P and C intake on male fertility (experiment 2). In our final experiment, we gave males the choice between alternate diets in four diet pairings to determine whether they regulate their intake of P and C to optimize sperm production, sperm viability and/or subsequent fertility.

2. Material and methods

(a) Experimental animals

Animals used in experiments were taken from a laboratory colony of *N. cinerea* with over 200 000 individuals housed in 10 large plastic containers (80 × 50 × 30 cm) in an incubator set at 28 ± 1°C and a 14 L : 10 D lighting regime. Colonies are fed weekly with dry rat chow and given water in two large test tubes (15 cm long, 4 cm diameter) plugged with cotton wool. To maintain genetic diversity, several hundred individuals are swapped between containers every two months when cleaned. Late instar nymphs were collected at random from each of these containers and sorted into two smaller containers (17 × 12 × 6 cm) by sex. These containers were checked daily and any newly eclosed adults removed. Newly eclosed females were kept as mating partners and housed in individual containers (11 × 11 × 3 cm), and fed an ad libitum supply of commercial rat chow and provided with water in a small test tube (8 cm long, 1.5 cm diameter) plugged with cotton wool. Newly eclosed males were housed in individual containers (20 × 10 × 10 cm) and haphazardly assigned to an experiment, and a diet (experiment 1 and 2) or a combination of diets (experiment 3) within experiments, on their day of eclosion. All experimental animals were maintained in a large, constant-temperature room set at 28 ± 1°C and a 14 L : 10 D lighting regime.

(b) Artificial diets and measuring diet intake

We made 24 artificial, dry diets that varied in P and C, as well as overall nutrition, based on the established protocol outlined by

Simpson & Abisgold [44]. The distribution of these diets in nutritional space can be seen in the electronic supplementary material, figure S1, and the composition of these diets in the electronic supplementary material, table S1. These represent the same 24 diets used by South *et al.* [45].

Each experimental male was given either one (experiment 1 and 2) or two (experiment 3) dishes of diet of measured dry weight on their day of eclosion, and diet was changed every 5 days for a total of 10 days post-eclosion (i.e. two feeding periods) when males were sexually mature. Food and water were provided in feeding platforms constructed by gluing the upturned lid of a vial (1.6 cm diameter, 1.6 cm deep) onto the middle of a Petri dish (5.5 cm diameter). Any diet that spilled during feeding was collected in the Petri dish and weighed. All diets were dried in an oven (Binder, model FD 115) at 30°C for 48 h before weighing. Feeding platforms containing diet were weighed before and after each feeding period using an electronic balance (Ohaus Explorer Professional, model EP214C). Before weighing, any faeces were removed from the diet and feeding platform using forceps. Diet consumption was calculated as the difference in dry weight of diet before and after feeding. This amount of consumed diet was converted to a weight of P and C ingested by multiplying by the proportion of these nutrients in the diet [45].

(c) Experiment 1: the effects of nutrient intake on sperm number and viability

To determine the effects of P and C intake on sperm number and viability, 15 males were established at random on each of the 24 diets (total $n = 360$ males) on their day of eclosion. Males were fed for 10 days and then mated to a virgin female of the same age. After mating, females were dissected and the spermatophore removed. The sperm-containing ampulla was detached from the spermatophore with fine forceps and crushed in 200 µl of cockroach ringer solution (150 mM NaCl, 3.1 mM KCl, 85.4 mM CaCl, 2.0 mM MgCl, 5 mM TES) using a pipette tip [40]. This sperm sample was mixed by hand for 1 min, and then a 10 µl sample was diluted in 500 µl of deionized water. This diluted sample was mixed by hand for a further 1 min, and six 5 µl subsamples were placed on slides with coverslips. To quantify the number of sperm produced, the subsamples were viewed under 10× magnification using phase contrast on a fluorescent microscope (Olympus, BX61) fitted with a digital camera (Olympus, DP70), and the total number of sperm in each subsample was counted and converted back to the original sample volume. In a subset of 20 males, our estimate of sperm number was shown to be highly repeatable using this sampling procedure (repeatability = 0.93, 95% CI: 0.89, 0.97) [46]. The mean number of sperm across these subsamples was used in all subsequent analyses.

To quantify sperm viability, 175 µl of the original sperm sample was stained using a commercially available live/dead sperm viability kit (Life Technologies, L-7011) following the instructions provided with the kit. Two 5 µl subsamples were placed on a slide with coverslip and viewed using an Olympus BX51 fluorescent microscope under 20× magnification. To measure the viability of sperm produced, the number of live (green) and dead (red) sperm on five haphazardly chosen fields of view of each subsample were counted (a total of 10) [34]. Sperm viability was calculated as the proportion of live sperm out of the total number of sperm counted. It is important to note, however, that this sperm viability assay may not provide a good indication of which sperm have the capacity to fertilize an egg, because even non-motile sperm can be categorized as live [47]. In a subset of 20 males, our estimate of sperm viability was shown to be highly repeatable using this sampling procedure (repeatability = 0.78, 95% CI: 0.64, 0.91). The mean number of sperm across these subsamples was used in all subsequent analyses.

(d) Experiment 2: the effect of nutrient intake on male fertility

To determine the effects of P and C intake on male fertility, 20 males were established at random on each of the 24 diets (total $n = 480$ males) on their day of eclosion to adulthood. Males were fed for 10 days and then mated with a virgin female of the same age. Females were then returned to their individual boxes and provided with an ad libitum diet of rat chow and water, and maintained until parturition. Water and food were replaced, and the container cleaned weekly. On the day of parturition, the number of babies produced by each female was recorded. A total of 44 females aborted their clutch during this experiment. As this incidence of clutch abortion was unrelated to the P and C intake of their mating partner (table 1), these females were excluded from subsequent analyses of male fertility.

(e) Experiment 3: measuring nutrient intake under choice

A total of 60 males were assigned haphazardly to one of four possible diet pairings ($n = 15$ per diet pair) that differ in both P : C ratio and total nutrition (%P + %C). The diet pairings we used (P : C ratio followed by total nutrition percentage in parentheses) were: pair 1, 5 : 1 (36%) versus 1 : 8 (36%); pair 2, 5 : 1 (36%) versus 1 : 8 (84%); pair 3, 5 : 1 (84%) versus 1 : 8 (36%); and pair 4, 5 : 1 (84%) versus 1 : 8 (84%). This corresponds to diets 2, 4, 22 and 24 in electronic supplementary material, table S1 and provides a broad coverage in nutrient space (electronic supplementary material, figure S1). Diet consumption and intake of P and C were measured over a 10-day period for each cockroach using the protocol described above.

(f) Statistical analysis

We quantified the linear and nonlinear (quadratic and correlational) effects of P and C intake on sperm number, sperm viability and male fertility using a multivariate response surface approach (outlined in the electronic supplementary material, text S1) [45]. Non-parametric thin-plate splines were used to visualize the nutritional landscape for each response variable and were constructed using the *Tps* function in the 'FIELDS' package in R (version 2.13.0).

We used a sequential model-building approach to determine whether the linear and nonlinear effects of P and C intake differed across our response variables (outlined in the electronic supplementary material, text S2) [45]. While this sequential approach provides a statistic test of the difference in magnitude of the linear and nonlinear gradients across response variables, it does not provide information on the direction of this difference in nutrient space. Thus, it is possible that response variables show differences in the magnitude of linear and nonlinear gradients, even though the optimal expression of these traits resides in a similar location in nutrient space. Consequently, we also calculated the angle (θ) between the linear vectors for the two response variables being compared as

$$\theta = \cos^{-1}\left(\frac{a \cdot b}{\| a \| \| b \|}\right), \tag{2.1}$$

where a represents the linear effects of P and C intake in the first response variable being compared, b represents the linear effects of these nutrients for the second response variable, $\| a \| = \sqrt{a \cdot a}$ and $\| b \| = \sqrt{b \cdot b}$. When $\theta = 0°$, the vectors are perfectly aligned, and the optima for the two response variables reside in the same

Table 1. The effect of protein (P) and carbohydrate (C) intake on sperm number and viability in male *N. cinerea* and the effect on the number of offspring produced by their female mating partner.

	linear effects		nonlinear effects		
response variable	P	C	P × P	C × C	P × C
sperm number					
gradient (95%CI)	0.18 (0.12, 0.25)	0.70 (0.63, 0.77)	−0.04 (−0.08, 0.00)	−0.30 (−0.33, −0.26)	0.51 (0.43, 0.59)
t_{359}	5.22	19.82	2.19	16.48	12.29
p	0.0001	0.0001	0.03	0.0001	0.0001
sperm viability					
gradient (95%CI)	−0.06 (−0.17, 0.05)	−0.03 (−0.14, 0.08)	0.00 (−0.08, 0.08)	−0.02 (−0.10, 0.05)	0.03 (−0.14, 0.19)
t_{359}	1.10	0.53	0.04	0.62	0.31
p	0.27	0.60	0.97	0.54	0.76
offspring number[a]					
gradient (95%CI)	0.14 (0.06, 0.22)	0.55 (0.47, 0.62)	−0.06 (−0.10, −0.02)	−0.22 (−0.26, −0.17)	0.35 (0.23, 0.47)
t_{435}	3.41	13.87	2.82	9.09	5.61
p	0.001	0.0001	0.005	0.0001	0.0001

[a]Females that aborted clutches ($n = 44$) were removed from the analysis. A randomization test, where females that aborted their clutch were assigned a value of 1 and those that did not were assigned a value of 0, showed that the incidence of clutch abortion was unrelated to the intake of P or C (P: 491/10000, $p = 0.10$; C: 2746/10000, $p = 0.55$; P × P: 6128/10000, $p = 0.77$; C × C: 9490/10000, $p = 0.10$; P × C: 940/10000, $p = 0.19$).

location in nutrient space, whereas $\theta = 180^\circ$ represents the maximum possible divergence between vectors. To determine the significance of θ, we estimated the 95% credible interval (CI) of this angle using a Bayesian approach implemented in the 'MCMCglmm' package of R. For each response variable (R), we ran a separate linear model ($R \sim \beta_1 P + \beta_2 C + \varepsilon$) using 400 000 Markov chain iterations with a burn-in of 20 000, a thinning interval of 25 and a relatively uninformative prior ($v = 0.02$), to create a posterior distribution of β. We used these distributions in equation (2.1) to generate 15 200 values for θ; the median of these values was used as our point estimate of θ, and the 2.5% and 97.5% quantiles used as our estimate of the 95% CIs. R code for this procedure is provided in the electronic supplementary material (text S3).

We used a paired t-test to compare the consumption of diets in each diet pair. As multiple comparisons were used (four in total), we corrected our significance level using a Bonferroni correction (adjusted $a = 0.0125$). We used a multivariate analysis of variance (MANOVA) to compare the total intake of P and C across diet pairs in our choice experiment (experiment 3). We used univariate ANOVAs to determine which nutrient contributed to the overall multivariate effect of diet pair and pairwise Tukey's honest significant difference (HSD) tests to contrast the total intake of nutrients across specific diet pairs. We calculated the point in nutritional space that individuals actively defend when given dietary choice, known as the regulated intake point, as the mean intake of P and C across these diet pairs [43].

3. Results

The intake of both P and C had clear linear and nonlinear effects on the number of sperm produced by a male (table 1 and figure 1*a*). Sperm number increased linearly with the intake of both nutrients, although it was over three times more responsive to the intake of C than P (table 1). The significant quadratic terms indicate a peak in sperm number (table 1), and inspection of the nutritional landscape shows that this peak occurs at high intakes of P and C, centred around a P : C ratio of approximately 1 : 2 (figure 1*a*). There was also a significant positive correlational gradient (table 1), providing further evidence that an increase in both nutrients increased sperm number. In contrast to sperm number, the intake of P and C did not significantly influence sperm viability (table 1).

Male fertility also increased linearly with the intake of P and C (table 1). The nutritional landscape for male fertility shows a high degree of similarity to the landscape for sperm number, with a peak occurring at high intakes of P and C in a ratio of approximately 1 : 2 (table 1 and figure 1*b*). Formal statistical comparison using a sequential model approach showed that the linear and correlational effects of P and C on sperm number and male fertility differed significantly (linear: $F_{2,789} = 5.29$, $p = 0.005$; correlational: $F_{1,783} = 4.60$, $p = 0.03$), but the quadratic effects of these nutrients did not (quadratic: $F_{2,785} = 0.59$, $p = 0.56$). The difference in linear gradients was due to the fact that sperm number was more responsive to C intake than male fertility ($F_{1,789} = 8.13$, $p = 0.004$), whereas both traits were equally response to the intake of P ($F_{1,789} = 0.64$, $p = 0.43$; table 1). Likewise, the difference in the correlational gradient was due to a stronger gradient for sperm number than for male fertility (table 1). Importantly, however, the peaks for sperm number and male fertility occur in the same region on the nutritional landscapes (figure 1*a*,*b*), as evidenced by the small angle (i.e. θ is close to 0°) between the two linear vectors ($\theta = 8.71^\circ$, 95% CI: 6.50°, 9.14°).

In each diet pair, males consumed significantly more of the high-C diet than the high-P diet (electronic supplementary material, figure S2). Accordingly, a MANOVA revealed that the intake of P and C differed significantly across diet

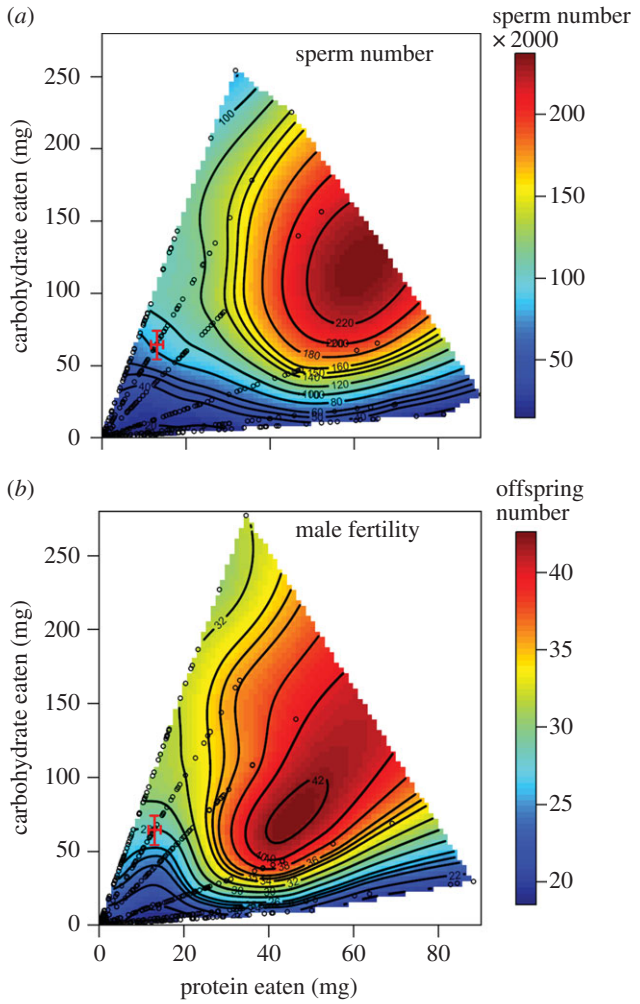

Figure 1. The nutritional landscapes for male (*a*) sperm number and (*b*) fertility (measured as the number of offspring produced by his mating partner) in *N. cinerea*. In each landscape, the red regions represent higher values of the male trait, whereas blue regions represent lower values of the male trait. The red symbol represents the regulated intake point (and 95% CIs) calculated in experiment 3. (Online version in colour.)

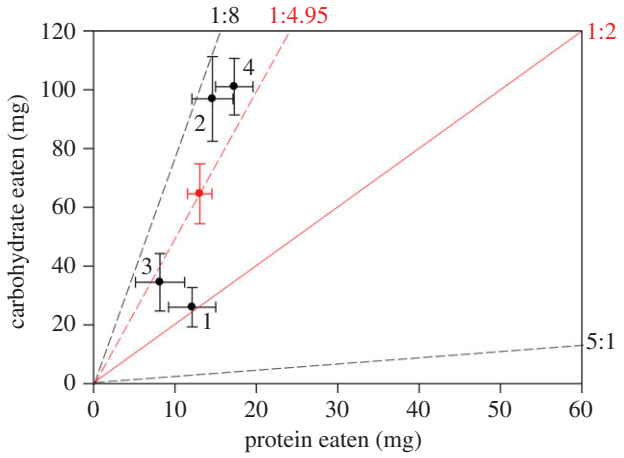

Figure 2. The mean (95% CI) intake of P and C by male *N. cinerea* when given dietary choice. The black symbols represent the mean intake of nutrients in each of the four diet pairs (denoted by diet pair number), whereas the red symbol represents the regulated intake point (mean of the four diet pairs). The dashed red line is the nutritional rail (a line in nutrient space that represents a fixed intake of nutrients) that passes through the regulated intake point (at a P : C ratio of 1 : 4.95). The solid red line is the nutritional rail that passes through the peak of the nutritional landscapes for sperm number and male fertility (at P : C ratios 1 : 2). The black dashed lines (at P : C ratios of 5 : 1 and 1 : 8) represent the outer nutritional rails along which males would feed if only eating one of the diets in the pair. (Online version in colour.)

pairs and univariate ANOVAs showed that both nutrients contributed to this difference (electronic supplementary material, table S2; figure 2). Although males clearly regulate their intake of P and C, Tukey's HSD pairwise contrasts revealed that the intake of these nutrients was not very tightly regulated (i.e. there were differences in nutrient intake across diet pairs; electronic supplementary material, table S2; figure 2). The regulated intake point was calculated at an intake of 13.06 (95% CI: 11.60, 14.55) mg of P and 64.61 (54.37, 74.22) mg of C, which equates to a P : C ratio of 1 : 4.95 (figure 2). This regulated intake point does not correspond with the peaks for sperm number or male fertility (figure 1), and therefore demonstrates that the regulation of P and C intake is not optimal for these traits.

4. Discussion

Here, we used nutritional geometry to examine the effect of P and C intake on ejaculate characteristics (sperm number and viability) and subsequent fertility in male *N. cinerea*. We found a clear effect of P and C intake on the number of sperm produced by a male, being maximized at a high intake of diets with a P : C ratio of 1 : 2. This change in sperm production with nutrient intake also had significant implications for male fertility, as the nutritional landscapes for sperm number and male fertility were closely aligned. In contrast, we found that sperm viability was not significantly influenced by the intake of P and C, suggesting that this trait is either not costly to maintain as an adult or is regulated by factors other than the intake of these nutrients. When given dietary choice, males showed a clear preference for the intake of C and regulated their intake of nutrients at a P : C ratio of 1 : 4.95. Collectively, these findings demonstrate that sperm production is costly and dependent on the balanced intake of P and C in *N. cinerea*, but males do not optimize sperm production when given dietary choice.

The intake of P and C has clear effects on sperm production in *N. cinerea*. Two lines of evidence suggest that sperm are costly to produce in *N. cinerea*. First, we show that sperm number increased with the overall intake of nutrients (and therefore calories or energy). Second, we show that sperm production was maximized at a P : C ratio of 1 : 2. Thus, it is not only a high intake of nutrients that is important for sperm production; the intake of P and C must also be balanced to maximize sperm production in *N. cinerea*. The finding that sperm production increases with the overall intake of nutrients is consistent with more resources being available to allocate to sperm production, and is in agreement with studies on Queensland fruitflies [27], red flour beetles [29], Indian meal moths [30] and *D. melanogaster* [28]. It contrasts, however, with studies on ladybirds [31] and Mediterranean fruitflies [32], which show a reduction in sperm number with increased nutrition. One possible reason for these opposing effects is the variety of dietary manipulations used in these studies. Unlike all these previous studies, we used holidic diets to precisely vary the P and C content of diets, and precisely measured the intake of these nutrients by males in our experiment. Using diets with imprecise or unknown nutritional composition and ignoring compensatory

feeding has the potential to greatly influence the intake of nutrients [48]. Thus, it is possible that the decrease in sperm number seen in ladybirds [31] and Mediterranean fruitflies [32] with higher diet quality may actually reflect a suboptimal intake of nutrients for sperm production in these species. Our work therefore clearly illustrates the value of using the GF approach to understand dietary effects on male ejaculates [43].

Our finding that sperm production is maximized at a high intake of diets with a P : C ratio of 1 : 2 is remarkably similar to the optima observed for female egg production in a range of insect species, including *D. melanogaster* (P : C ratio = 1 : 2 [49,50]), Queensland fruitflies (P : C = 1 : 2.3 [51]; P : C = 1 : 1 [52]) and field crickets (P : C = 1 : 1 [53]). This ratio, however, contrasts the optima shown for male traits used in pre-copulatory sexual selection, such as nightly calling effort in the black field cricket (*Teleogryllus commodus*), which requires a much higher intake of C to fuel this energetically costly behaviour (P : C ratio = 1 : 8 [53]). In *N. cinerea*, male–male competition and female mate choice are mediated by the same three male sex pheromones (3-hydroxy-2-butanone, 4-ethyl-2-methoxyphenol and 2-methylthiazolidine [54]), and the expression of these pheromones and subsequent attractiveness is also optimized at a P : C ratio of 1 : 8 [45] (see the electronic supplementary material, text S4 for a detailed comparison of our feeding methodologies [45]). Collectively, these findings have two important implications for the evolution of sperm number in *N. cinerea*. First, it shows that male and female gametes share a common requirement for P to optimize their production. While the effect of P intake on sperm production is largely unknown in insects, it is well documented in mammals [55]. Second, it demonstrates that the combination of nutrients that is optimal for sperm production is suboptimal for pre-copulatory attractiveness, and vice versa. This suggests that sperm production is traded against attractiveness, and that the intake of P and C regulates this trade-off.

In our study, we quantified male fertility as the number of offspring produced by a randomly assigned mating partner in her first clutch. As such, our measure of male fertility is not only determined by the male, but also has the potential to be influenced by the female. Despite this, we found that the nutritional landscapes for male fertility and sperm number were closely aligned, with both traits being maximized at a high intake of diet with a P : C ratio of 1 : 2. This finding strongly suggests that sperm number is a major determinant of male fertility in *N. cinerea* and agrees with previous experimental work on this species [35,39]. For example, Montrose *et al.* [35] showed that ampulla size decreases significantly across four consecutive matings in *N. cinerea*, and that this pattern was almost perfectly matched by a decline in the number of offspring produced by a mating partner in her first clutch as well as over her lifetime. However, our experimental design means that we cannot completely rule out that some of the variation in the nutritional landscape for male fertility is influenced by contributions from the female or other constituents of the ejaculate. For example, female *N. cinerea* are known to prolong gestation and produce more female-biased clutches [42] after mating with more attractive males. This is unlikely, however, to have a major impact on the nutritional landscape for male fertility, because male attractiveness is maximized on more C-biased diets compared with fertility (P : C ratio of 1 : 8 versus 1 : 2 [45]). Other constituents of the ejaculate also have the potential to influence the nutritional landscape for male fertility. For example, male *D. melanogaster* are more successful in sperm competition on diets containing medium to high amounts of protein, probably owing to the increased production of accessory gland proteins [56]. Accessory glands in cockroaches are known to produce a cocktail of chemicals, including urea [57] and trehalases [58], but whether these substances influence male fertility or their production varies with nutrition remains to be tested.

Our work clearly shows that males can optimize both sperm production and subsequent fertility by consuming a high intake of diet with a P : C ratio of 1 : 2. Yet, when given the opportunity to choose between alternate diets, males actively regulated their intake of nutrients at a P : C ratio of 1 : 4.95. This provides clear evidence that male *N. cinerea* do not regulate their intake of P and C to optimize these traits, and raises the important question: why do males prefer a C-biased diet when this comes at a direct cost to sperm production and fertility? One possible answer is that males preferentially consume more C to maximize their pre-copulatory attractiveness [45]. Given the relatively low risk of sperm competition in *N. cinerea*, males may maximize their reproductive success by mating more frequently rather than maximizing the number of offspring produced from each mating. Furthermore, a high intake of C is known to extend lifespan in a range of insect species [49,51–53], so it is possible that preferentially consuming more C also increases the lifetime reproductive success of males. Interestingly, the observed regulated intake point (P : C = 1 : 4.95) was almost perfectly intermediate between the P : C ratio maximizing sperm production and fertility (P : C = 1 : 2) and that maximizing sex pheromone production and pre-copulatory attractiveness (P : C = 1 : 8 [45]). It is therefore possible that males regulate their intake of nutrients to balance the trade-off between these important pre- and post-copulatory traits, although more work is clearly needed to test this idea.

In contrast to sperm number and male fertility, we found little effect of P and C intake on sperm viability in *N. cinerea*. This result is surprising given that previous work on this species suggests that sperm viability is likely to be costly to maintain [35,38]. For example, males increase the viability of sperm in their ejaculate in response to competitive interactions with other males [38], and sperm viability also decreases significantly across consecutive matings [35]. Similar costs have also been shown in the Pacific field cricket (*Teleogryllus oceanicus*), where males adjust the viability of sperm in their ejaculate in response to a variety of cues that signal the risk of sperm competition [59], and sperm viability is also traded against immune function [24]. However, despite the important role that sperm viability plays in biasing the outcome of sperm competition in insects [13], only a single study has examined the effect of diet on sperm viability, and found that pollen restriction had little effect on sperm viability in honeybees [33]. There are a number of possible reasons why the intake of P and C did not influence sperm viability in *N. cinerea*. First, it is possible that sperm viability is determined more by a male's genotype than by the quality of the nutritional environment experienced as an adult. However, this explanation is unlikely in *N. cinerea*, because the heritability of sperm viability ($h^2 \pm$ s.e.: 0.15 ± 0.15) is much lower than for other ejaculate characteristics, including sperm number, which showed a clear response to P and C intake in our study (0.40 ± 0.31 [34]). Second, it is possible that any effect of nutrient intake on

sperm viability only occurs during juvenile development. In *N. cinerea* [40], testis development, and presumably the capacity to manufacture viable sperm, occurs during the nymph stages. As this was before males were randomly assigned to diets in our study, it could explain the observed lack of responsiveness of sperm viability to adult nutrition. Finally, key nutrients other than P and C may be responsible for regulating sperm viability in *N. cinerea*. In vertebrates, it is well established that various micronutrients are essential for the production of viable sperm, including trace minerals (e.g. selenium and zinc), vitamins (e.g. vitamins A, E and D) and essential fatty acids (e.g. phospholipids; reviewed in [55]). Unfortunately, we still know very little about the role that these micronutrients play in regulating sperm viability in insects.

In conclusion, our study shows that sperm production is costly and that a balanced intake of P and C is required to maximize this important determinant of male fertility in *N. cinerea*. The next obvious question to address is: exactly how costly is sperm production? Answering this question requires an integrated approach that examines the cost of sperm production in relation to other important life-history traits. While we have not taken this approach here, our study does highlight the potential utility of the GF in addressing this fundamental question. For example, by using the same geometrical array of holidic diets, we now know that the optimal balance of P and C intake is different for sperm production and pre-copulatory attractiveness in male *N. cinerea*. Our future work therefore plans to continue using the GF approach to further improve our understanding of how costly sperm production is in this species.

Data accessibility. All data are provisionally stored at doi:10.5061/dryad.n40m6.

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