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Nutritional geometry of paternal effects on embryo mortality

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Well-established causal links exist between maternal nutritional deficits and embryo health and viability. By contrast, environmental effects operating through the father that could influence embryo mortality have seldom been examined. Yet, ejaculates can require non-trivial resource allocation, and seminal plasma components are increasingly recognized to exert wide-ranging effects on females and offspring, so paternal dietary effects on the embryo should be expected. We test for effects of varying levels of protein (P), carbohydrate (C) and caloric load in adult male diet on embryo mortality in *Drosophila melanogaster*. We demonstrate that macronutrient balance and caloric restriction exert significant effects, and that nutritional effects are more impactful when a prior mating has occurred. Once-mated males produced embryos with marginally elevated mortality under high-caloric densities and a 1 : 8 P : C ratio. In contrast, embryos produced by twice-mated males were significantly more likely to die under male caloric restriction, an outcome that may have resulted from shifts in ejaculate quality and/or epigenetic paternal effects. Body nutrient reserves were strongly and predictably altered by diet, and body condition, in turn, was negatively related to embryo mortality. Thus, sire nutritional history and resultant shifts in metabolic state predict embryo viability and post-fertilization fitness outcomes.

1. Introduction

Offspring typically require considerable investment of time, energy and other resources to succeed [1]. Because females produce large and nutritious eggs, they by definition invest more than males into gamete production, and in many animal species maternal care continues to be substantial over the course of offspring growth and development [2]. Consequently, variation in the health and nutritional state of the mother can exert a profound influence on offspring fitness-related phenotypes. Embryo mortality is of considerable interest, as it can be a crucial determinant of reproductive failure in a variety of animal species [3–7], including humans. For example, clinical studies of human pregnancy loss estimate that embryonic mortality prior to implantation may account for a striking 30% of conceptions [8]. Research into maternal transmission of nutritional and other environmental effects to the embryo is both abundant and diverse [9–11].

In contrast, the role of paternal dietary effects on embryo viability has been a neglected area of study. This research imbalance is perhaps unsurprising, given that sperm are tiny and lack a nutritive component, and that ejaculates have traditionally been assumed to be inexpensive to produce [2,12], but see [13]. Moreover, males are often also emancipated from many forms of progeny provisioning and care, which is a hallmark of polygynous species where males transfer nothing to the female other than an ejaculate at mating [1,14]. However, ejaculates are composed of both sperm cells and seminal fluid, the latter containing a complex mixture of male accessory gland secretions [15], components of which

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are increasingly recognized to affect certain aspects of female physiology and behaviour, and in shaping offspring phenotypes [16–18]. In mice, seminal plasma components maintain sperm viability and integrity, as well as influence normal embryo development and offspring health [19]. Moreover, ejaculate production and the maintenance of reproductive tissues are in fact not cost free, and can require non-trivial resource investment [20–23], often at the expense of other fitness traits [24,25]. Thus, ejaculatory traits should often be sensitive to male nutritional history, for which there is some empirical support [26,27], although such an effect is not always found [26,28].

Recently, studies have begun to examine effects of major macronutrients in a geometric framework [29] on male reproductive traits, such as testes mass and epididymal sperm counts in mice [23], sperm competitiveness in flies [30] and calling effort in crickets [31]. Macronutrients are an essential component of an organism's nutritional environment, and there is promise of applying the geometric framework approach toward understanding the relative roles of macronutrient balance and calories on male factor infertility, similar to its successful deployment for major life-history traits in females including lifespan and fecundity [32–35]. At present, there are next to no data available concerning macronutrient effects on embryo mortality exerted through adult males, limiting our knowledge of the scope of potential effects of adult diet on post-fertilization fitness outcomes. In one study, sperm number in cockroaches, *Nauphoela cinerea*, increased with intake of P and C, whereas sperm viability was not affected by macronutrient intake [26]. Hatching success of individual eggs was not quantified in this study, although approximately 9% of females aborted their clutches. Clutch abortion, however, was not related to male nutrient intake, suggesting that abortion rate is related to maternal condition in this cockroach species.

Here, we systematically varied dietary levels of P (as yeast) and C (as sucrose or fructose) using agar-based substrates [35] to deliver the experimental diets to adult male *Drosophila melanogaster*. Whereas sucrose is commonly used as source of C in such feeding studies [30,33–35], we expanded the design to compare the effects of sucrose and fructose; both sugars are present in the fruit substrates on which larvae and adults feed in nature [36,37], and they can have differential effects on adult fly metabolic, life-history, and post-mating behavioural phenotypes [38–40]. Males in our study were maintained on their distinct diets for 17 days, and then mated consecutively to two virgin females, designated A and B. After mating, females were allowed to lay eggs under standard conditions for 24 h, and the proportion of fertilized eggs that died estimated the embryonic death rate. With these data we modelled interactive effects of dietary macronutrient concentrations, as well as total energy content of the food, on the viability of embryos the test males sired. Concomitant shifts were also measured in three major indices of male nutrient reserves (*viz*. total body protein, lipids and glycogen), thereby integrating information about physiological state into our assessment of paternal nutritional effects through to the embryo. Sires were given *ad libitum* access to a water source during feeding, thus decoupling dietary treatment from the potential physiological consequences of dehydration: we were concerned that dehydration could confound assessment of paternal dietary effects on measured responses, a complication demonstrated previously for fly lifespan [41].

2. Material and methods

(a) Source of males

Males were wild-type Canton-S *D. melanogaster* Meigen used by Lee *et al*. [34]. Culture procedures are provided in the electronic supplementary materials and methods. Males were sourced from culture vials previously seeded by allowing 10 randomly chosen sexually mature females (plus 10 males) to lay eggs for 24 h. Adult flies that emerged from these density-controlled vials were harvested as virgins within 5 h of emergence under light CO_2 anaesthesia. Virgin males were randomly allocated to experimental diets. The experiment consisted of two replicates, with start times staggered 5 days apart.

(b) Experimental diets

Diets were prepared by systematically varying yeast and sugar concentrations in order to achieve the desired array of protein (P) and carbohydrate (C) ratios and caloric densities. The experiment used two sugars, sucrose and fructose, as the main source of carbohydrate. For each sugar, we used five P : C ratios of 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32 at each of three caloric densities (total P + C) of 100, 200 and 400 g l^{-1}. The caloric content of protein per unit mass is equivalent to carbohydrate [42], so we consider total P + C to represent caloric load. Protein was entirely derived from hydrolysed yeast (MP Biomedicals, catalogue no. 0210330405). Macronutrient concentrations were calculated based on an analysis of the hydrolysed yeast conducted by FeedTest Laboratory (Victoria, Australia), indicating 62.1% protein and 1.9% total carbohydrate (free sugars + starch). Thus, our experiment consisted of 15 experimental diets × 2 sugar types = 30 total diets for each of two overlapping replicates. Details on diet preparation are provided in the electronic supplementary materials and methods.

(c) Feeding protocol

Diets were delivered to test males in vials by allowing them to feed from the surface of the food [35]. Test males were maintained on their respective diets for 17 days, at a density of 10 males per vial. There was one vial per diet per replicate, and males were transferred to fresh food vials every 2 days. In replicate 1, two males were lost during transfer to fresh vials, and one male was injured during transfer and excluded from the experiment. In replicate 2, one male was lost during transfer, and one male was injured and, therefore, excluded. No males died during experimental feeding.

(d) Mating assay

In each replicate, matings were conducted at 26–27°C. Before lights were turned on, males were individually transferred under dim red light from feeding vials into numbered mating vials containing a 7 ml agar substrate (composed of 0.5% agar plus inhibitor). Five males per diet were used, so that there was a total of 150 mating vials. Mating vials were lined up in random sequence with respect to treatment along a desktop, and males were allowed to acclimate to mating vial conditions for 60 min in the dark. When lights were turned on at approximately 08.00 h, a 5-day-old virgin female was gently aspirated into each vial sequentially. All females had been held since emergence in yeasted cornmeal vials. In this way, female age and nutritional status were tightly controlled, limiting variation in maternal condition and embryo age at deposition [43]. Each mating vial was monitored until a copulation occurred or for a maximum of 2 h. Within 15 min of the end of a male's first copulation, the male was transferred to a fresh mating vial containing another 5-day-old virgin female. A male's first and second females are referred to as females A and B, respectively. Thorax length, as an estimate of body size, was measured for all females that mated. For each vial, we recorded the time at which (1) the

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female entered the vial, (2) copulation began, and (3) the pair separated. Copulation duration was taken as the time from onset of copulation to separation.

(e) Embryo death rate and total eggs laid

All mated A and B females were transferred to an oviposition vial within 3 h of copulation. Females were allowed to lay eggs in a first vial (Vial 1) for 24 h, and then transferred to a second oviposition vial (Vial 2) for an additional 24 h of egg laying. Oviposition vials contained grape-agar substrate to promote egg deposition and to provide a high-contrast background against which eggs could readily be examined. Embryo death rate was quantified for eggs deposited into Vial 1. For Vial 2 we also counted eggs deposited, but did not quantify the embryo death rate because of time constraints.

To quantify embryo death rate, we counted total eggs deposited by females under a stereomicroscope, and then incubated the vials for a minimum of 24 h at 25 ± 1°C and a 12 h (dark) : 12 h (light) photoperiod to allow eggs to hatch; in *D. melanogaster* embryogenesis lasts ≈22 h at 25°C [44]. Eggs are fertilized in the uterus, and when newly fertilized eggs are deposited they are homogeneous milky-white in appearance. Over the course of development, fertilized eggs undergo distinct and discernable morphological changes. Following the 24 h incubation period, all hatched and unhatched eggs were counted. Each unhatched egg was examined (at 60×) in the position it was laid, or carefully lifted from the food medium using a dull dissecting probe and put on its side. We used morphological criteria [44,45] to determine whether it had undergone development to confirm that the egg had been fertilized and the embryo had died. Mid-stage embryos were identified by a darkening of the inner region and the appearance of segment formation, and late-stage embryos were identified by the presence of prominent segmentation of the cuticle, trachea and mouthparts [44,45]. When structures could not be unambiguously discerned, the unhatched egg was placed in a drop of 1% physiological saline on a microscope slide and re-examined. If still no segmentation could be discerned, the egg was deemed unfertilized. After this first count, all vials were incubated for an additional 24 h under conditions described above, and unhatched eggs were each examined a second time to verify they were not viable (none were). Thus, the unfertilized category contained oocytes that were either truly unfertilized plus any that had been fertilized but that had died prior to mid-stage development (i.e. before any segment formation could be discerned, see above). Our analyses excluded this unfertilized category of eggs. In other words, our analyses considered variation only in the proportion of fertilized eggs that died from the mid-stage of development and beyond, and thus, by potentially excluding some early stage embryos that died, likely *under*estimated the true embryonic death rate somewhat. Of the total number of unhatched oocytes from A and B females, the proportion that fell into the unfertilized category was 0.170 and 0.182, respectively.

Prior to statistical modelling, we excluded all data for which females laid 0 or 1 egg. Among A females across replicates, 15 and 32 flies laid 0 and 1 egg, and among B females, 10 and 21 females laid 0 and 1 egg, respectively. Among A and B females, the average number of eggs per female from which we determined the embryo death rate was 28.8 (s.d. = 8.43, range 5–58, $n = 237$) and 29.1 (s.d. = 8.00, range 3–56, $n = 220$), respectively. Total eggs laid for a given female was calculated by summing the number of eggs she laid between the two oviposition vials.

(f) Nutritional indices of male body condition

While the males selected for the mating assay were acclimating to mating vial conditions (see above), the remaining flies from each diet were frozen and later assayed for whole-body lipids, glycogen and protein; details are provided in the electronic supplementary materials and methods. All measured body nutrient quantities were normalized to dry weight, and expressed as micrograms of body nutrient per milligram of dry weight. We calculated total *nutrient* reserves by summing across the three nutritional indices. The yield from the complete oxidation of triacylglycerols is about 9 kcal g^{-1} (38 kJ g^{-1}), and about 4 kcal g^{-1} (17 kJ g^{-1}) for carbohydrates and proteins [46]. Total *energy* reserves (kJ mg^{-1} fly dry weight) was calculated by first converting the quantity of each nutrient to kilojoules of energy using these conversion factors, then summing across indices.

(g) Statistical analyses

To model responses of our focal traits over diet macronutrient (protein and carbohydrate) space, we used generalized additive models (GAMs) [47] with the mgcv package [48] of the R language [49]. 2D response surfaces were visualized using thin-plate splines in R. Regression analyses were conducted in JMP [50] to assess relationships between nutritional indices of condition and embryo death rate; embryo death rate was arcsine(sqrt)-transformed prior to analyses. Details pertaining to GAM construction, model comparisons, and outlier assessment are provided in electronic supplementary materials and methods.

3. Results

(a) Paternal dietary effects on embryo mortality

For each of the males' two mates, we first compared response surfaces for embryo mortality corresponding to the different sugar types. For A females (males' first mates), sugar surfaces differed significantly (likelihood-ratio test (LRT), deviance: 28.72, $p = 0.003$). Consequently, macronutrient effects were evaluated using GAMs separately by sugar. For both sucrose and fructose, there were significant P × C interaction effects on embryo mortality ($p = 0.001$ and 0.005, respectively, electronic supplementary material, table S1). Visual comparison of the surfaces (figure 1*a*,*b*) showed that although peak mortality was shifted between the sugars, the shift occurred along the same isocaloric band of highest caloric density (400 g l^{-1} P + C). For sucrose, mortality was greatest at low P : C ratios (peaking at 1 : 8, figure 1*a*), whereas for fructose, the peak occurred at a 1 : 2 P : C ratio, at the far-right top corner of nutrient space (figure 1*b*).

For B females (males' second mates), sugar surfaces also differed significantly (LRT, deviance: 47.23, $p = 5.71 \times 10^{-06}$), so data were likewise analysed separately by sugar. For sucrose, there was a significant P × C interaction ($p = 0.00005$), whereas this interaction was not detected for fructose ($p = 0.491$) (electronic supplementary material, table S2; figure 1*c*,*d*). In notable contrast to A females, embryo mortality on the sucrose diets was highest at lowest caloric densities (100 g l^{-1} P + C), but likewise peaked at a 1 : 8 P : C ratio (figure 1*c*). Embryo mortality was lowest under dietary conditions of highest C and lowest P (blue areas of figure 1*c*,*d*). Thus, although the two sugar surfaces for B females differed significantly, they nevertheless showed broadly similar topographies, notably in terms of the location of lowest embryo mortality; lowest mortality in both cases was promoted by high-energy, low P : C ratio, diets.

To explore the caloric density effect further, we constructed a separate GAM for each sugar type, and entered caloric density as an additional explanatory term. Models were simplified by dropping non-significant covariates from previous models (*viz*., female thorax length and copulation duration). In all cases where previously we detected significant P × C effects, these significant effects persisted upon the

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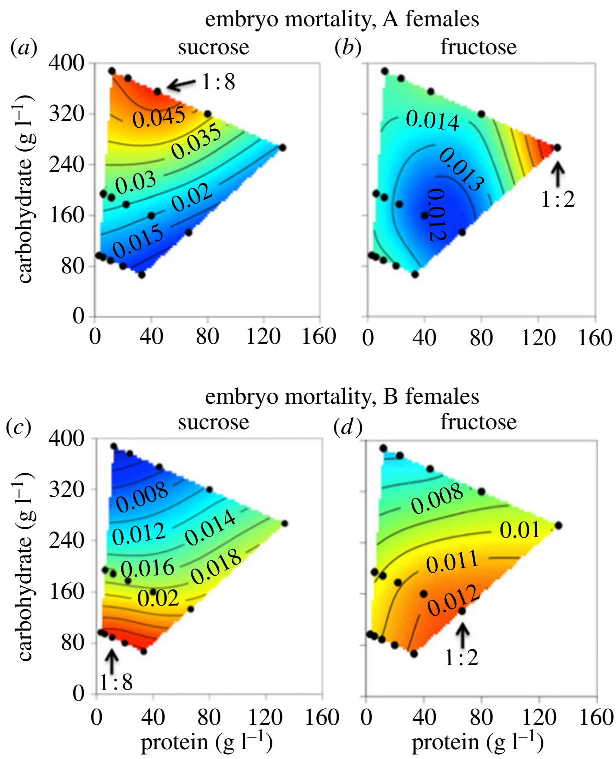

Figure 1. Responses of embryo mortality (proportion fertilized eggs that died) for (*a*,*b*) A females and (*c*,*d*) B females in 2D nutritional space. Surfaces are presented separately by sugar type, sucrose and fructose. Dark points in the figures represent individual diets, for which mean samples sizes are as follows (mean, s.d., range): (*a*) 7.60, 1.24, 4–9; (*b*) 8.07, 1.28, 5–10; (*c*) 6.87, 1.85, 4–10; (*d*) 7.60, 1.50, 5–10. Red regions represent elevated values of embryo mortality and blue regions are lower values of embryo mortality. Arrow in each panel identifies location of maximal embryo mortality.

addition of caloric density (table 1). For B females, in addition to the significant effect of the P × C interaction, there was also a significant effect of caloric density of embryo mortality: mean probability of embryo mortality decreased with caloric density (0.0167 (100 g l^{-1}), 0.0138 (200 g l^{-1}) and 0.00820 (400 g l^{-1})), as could be predicted from the related surface plot especially for sucrose (figure 1*c*).

Thus, these results demonstrate significant consequences of both macronutrient ratio and caloric density in the diet of sires for embryo mortality. The pattern of embryo mortality expressed in 2D nutritional space showed that macronutrient effects in A females was essentially in reverse configuration to that for B females, which was particularly evident for the sucrose-based diets. A calorically impoverished paternal diet increased embryo mortality, but only after males had had a previous mate.

(b) Dietary effects on indices of body condition

We tested for effects of the above nutrient regimen on male body condition, and found that varying levels of P and C, and the P × C interaction, exerted strongly significant effects (electronic supplementary material, table S3), consistent with previous work with this species [35]. Males accumulated greatest lipid reserves on low P and high C diets, and were leanest at high P (electronic supplementary material, figure S1a,b). For body protein reserves, there likewise were pronounced effects of dietary P and C, although the P × C interaction did not reach statistical significance for fructose (electronic supplementary material, table S3). Response surfaces for body protein were approximately reversed compared to those for lipids: body protein increased sharply with increasing dietary P (electronic supplementary information figures S1c,d). Protein reserves were lowest when dietary P and C were both in lowest abundance. Glycogen reserves, in turn, were primarily affected by C, as no significant effects of P or the P × C interaction were detected (electronic supplementary material, table S3). Body glycogen reserves increased with increasing C (electronic supplementary material, figures S1e,f). The effect of C on glycogen was significant for the sucrose-based diet (electronic supplementary material, table S3).

(c) Indices of body condition and embryo mortality

We tested whether the diet-induced changes in body nutrient reserves in turn predicted embryonic death rate. Given the significant effect of caloric restriction on embryo mortality described above for B females (see Results §3(a)), we predicted an inverse relationship between male body condition and the mortality of embryos deposited by these females. Although none of the six individual indices of sire condition significantly predicted embryo mortality (electronic supplementary material, table S4), all relationships were negative, and their mean was significantly different from zero (mean $\hat{\beta}$ (s.e.) = −0.003554 (0.001287), $t = -2.7604$ testing H_o: mean $\hat{\beta} = 0$, two-tailed $p = 0.040$). For A females, in contrast, four of the six regression coefficients were negative (and none were significant, all *ps* > 0.3), and the mean did not differ significantly from zero (mean $\hat{\beta}$ (s.e.) = 0.0009149 (0.001195), $t = 0.7656$, testing H_o: mean $\hat{\beta} = 0$, two-tailed $p = 0.479$).

We created an index of total nutrient reserves to capture variation in overall body condition of sires, and regressed embryo mortality on this index. Whereas the relationship was not significant for fructose (electronic supplementary material, table S4; figure 2*a*), it was significantly negative for sucrose (electronic supplementary material, table S4; figure 2*b*). A formal check for outliers identified two potentially influential data points identified with arrows in figure 2*b* (using the conservative criterion of Cook's $D > 0.5$, as all data had D values < 1.0). Removal of these data did not alter the regression conclusions (figure 2*c*). We also examined the relationship between total energy reserves, and found that it was likewise significantly negatively related to embryo mortality (electronic supplementary material, table S4)—males with fewest metabolic reserves sired embryos with greater average probability of death. For A females, relationships between total nutrient reserves or total energy reserves and embryo mortality were not significant, either for sucrose or fructose (*ps* > 0.5).

(d) Dietary effects on total fecundity of females

We tested for responses in female reproductive output to male dietary treatments, and found no significant effect of dietary P, C or the P × C interaction, on the total number of eggs deposited by either A or B females (electronic supplementary material, table S5). These results were consistent between the sugars.

4. Discussion

The main findings of our study are that varying macronutrient levels and caloric density of adult male diet significantly and

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Table 1. GAM results for embryo mortality with the addition of caloric density as an explanatory term. Terms that are significant or close to the chosen significance level are given in bold.

A females				
sucrose				
parametric terms	**estimate**	**s.e.**	***z***	***p***
Cal.density	−62.43	43.18	−1.446	0.148
smooth terms	**edf**	**Ref.df**	χ^2	***p***
s(prop.P)	1.000	1	2.089	0.148
s(prop.C)	**4.000**	**4**	**8.995**	**0.062**
s(prop.P, prop.C)	**3.389**	**6**	**14.588**	**0.002**
fructose				
parametric term	**estimate**	**s.e.**	***z***	***p***
Cal.density	37.02	31.90	1.160	0.246
smooth terms	**edf**	**Ref.df**	χ^2	***p***
s(prop.P)	2.577	2.791	1.893	0.511
s(prop.C)	1.000	1.000	1.347	0.246
s(prop.P, prop.C)	**3.870**	**6.000**	**12.031**	**0.004**
B females				
sucrose				
parametric term	**estimate**	**s.e.**	***z***	***p***
Cal.density	$\mathbf{-3.70 \times 10^{-3}}$	$\mathbf{1.39 \times 10^{-3}}$	**−2.658**	**0.008**
Replicate	**−0.733**	**0.300**	**−2.449**	**0.014**
smooth terms	**edf**	**Ref.df**	χ^2	***p***
s(prop.P)	1.43×10^{-4}	1.64×10^{-4}	0.00	0.995
s(prop.C)	1.00	1.00	1.279	0.258
s(prop.P, prop.C)	**2.068**	**3.00**	**17.261**	$\mathbf{3.18 \times 10^{-5}}$
fructose				
parametric term	**estimate**	**s.e.**	***z***	***p***
Cal.density	0.500	9.8471	0.051	0.960
smooth terms	**edf**	**Ref.df**	χ^2	***p***
s(prop.P)	1.000	1	0.003	0.960
s(prop.C)	1.000	1	0.003	0.959
s(prop.P, prop.C)	4.876×10^{-5}	6	0.000	0.423

independently affected probability of embryo mortality, and that, strikingly, these effects depended on male mating order. 2D surfaces depicting P × C effects on embryo mortality showed that responses were essentially in flipped configuration between a male's first and second females, and this effect was particularly evident for sucrose-based diets. Macronutrient concentrations exerted pronounced effects on all indices of male body condition, and body condition, in turn, predicted embryo mortality—males in the worst condition sired embryos with greater probability of death, and this effect was likewise restricted to when a previous mating event had recently occurred.

In all cases where significant P × C effects on embryo mortality were detected, the addition of caloric density as an explanatory term into the statistical models did not abolish the significant P × C interaction. This outcome suggests that the interactive effects of macronutrient concentrations modulate rate of embryo mortality independently of caloric density. Indeed, caloric density was itself also significantly negatively related to embryo mortality, but only among B females. In fact, for sucrose-based diets, embryo mortality among B females was approximately two fold greater on the most impoverished diets (100 g l^{-1}) relative to the most enriched ones (400 g l^{-1}).

The likely cause(s) of these results include diet-induced alterations to sperm integrity, seminal fluid composition or both. For example, dietary treatments that contribute to the development of metabolic syndrome in humans and mice can alter sperm characteristics, such as by causing DNA damage as nicks and breaks, and epigenetic modifications; both types of DNA alterations have the potential to compromise embryo viability and fetal development [51,52]. However, the facts that both diet and body condition effects were detected only

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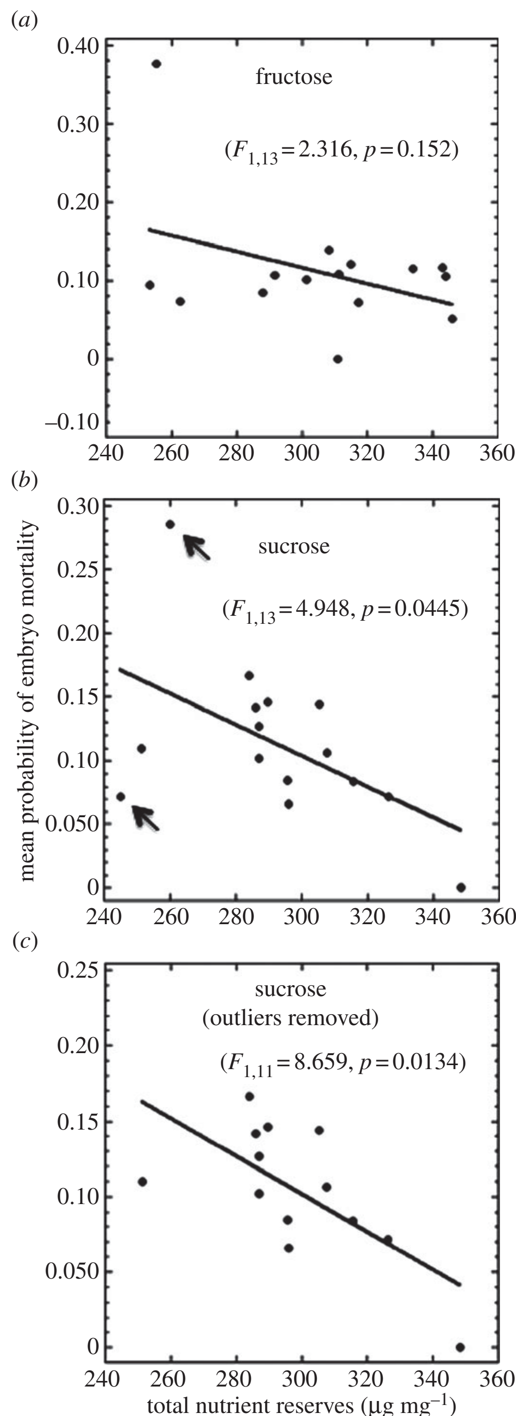

Figure 2. Relationships between mean probability of embryo mortality and total male nutrient reserves for (*a*) fructose-based diets and (*b*) sucrose-based diets. The significant negative relationship in the case of sucrose persisted upon the exclusion of two potentially influential outliers (*c*); excluded data are identified with arrows in panel b (top and bottom points, Cook's $D = 0.68$ and 0.86, respectively). Each point in panels represents $n = 4$ male flies (two flies per experimental replicate).

in twice-mated sires in our study points to shifts in seminal fluid composition also as a likely causal factor, because these results are consistent with patterns of seminal fluid depletion across successive matings. Previous work with *Drosophila* and other species has shown that males become depleted of seminal fluid components across successive copulations [53,54], and that males can strategically allocate seminal fluid products to females [55,56]. Indeed, in *D. melanogaster*, some accessory gland proteins (Acps) can deplete by as much as 30% in a single mating [54]. These observations suggest that seminal fluid production is under resource-based constraints and should be sensitive to diet. Moreover, whereas seminal fluid reserves in *Drosophila* and other species decline across subsequent copulations, they also do so more steeply than sperm supplies [53,57], which also fits with our results in that we failed to detect significant effects of male diet on female fecundity—we would have expected females to have deposited fewer eggs had they carried reduced sperm supplies [58].

The known functional properties of seminal fluid in *Drosophila* and other animals, further implicate seminal fluid in contributing to observed dietary effects. In *Drosophila*, seminal fluid comprises secretory products of the accessory glands, ejaculatory duct and ejaculatory bulb [59], and like in other animals, several Acps in the seminal fluid have been shown to exert a diversity of effects [15,60]. In *Drosophila*, Acps exert effects on female behaviour, physiology and sperm use, and of particular relevance to our study, potentially on sperm integrity and embryo viability as well [59,61]. For example, after copulation, the majority of Acp62F in *D. melanogaster* remains in the female reproductive tract including within the sperm storage organs, suggesting that it may play a role in the protection of sperm, perhaps by preventing attack by proteases [62]. Antimicrobial peptides that have been identified in the ejaculate may also protect sperm, eggs as well as the embryo from microbial attack [15,63,64]. Such peptides may play a protective role by coating the surface of the fertilized egg, as has been suggested for the medfly, *Ceratitis capitata* [65].

Thus, although the dietary effects on embryo mortality may have resulted from nutritionally deprived males being unable to transfer optimal quantities of specific seminal plasma components, potentially more complex factors may have also been at play. For example, overproduction of reactive oxygen species (ROS), either within the seminal fluid male or testicular environment, could have damaged the sperm genome/epigenome, thereby contributing to the responses we observed. In a recent study, when larval *D. melanogaster* were fed diets containing a range of sucrose concentrations (0.25–20%, w/v), adults from the lowest concentration exhibited elevated levels of oxidized lipids and protein, in addition to elevated superoxide dismutase activity [66]. It was suggested that the redox imbalance associated with carbohydrate restriction might actually be causally related not to carbohydrate levels per se, but to associated adverse effects of protein overconsumption [66]. Under conditions of inadequate dietary carbohydrate, animals may over-consume protein to reach their carbohydrate 'intake target' [34,67], and consequently incur ROS-induced damage to cellular constituents [66]. This effect on sperm possibly contributed to the elevated embryo mortality we observed with decreasing caloric density and body condition. However, if the elevated mortality we detected had been driven by sperm damage, we would have expected patterns of mortality expression over nutrient space to be more or less congruent between A and B females, which it was not. It nevertheless remains possible that changes in embryo mortality occurred through the *combined* effects on seminal fluid and sperm, as the seminal fluid itself may possess antioxidant properties, and in this way protect sperm DNA from ROS attack [68]. In golden hamsters, *Mesocricetus auratus*, antioxidant enzymes within the male accessory gland fluid protect the sperm from oxidative damage; without this protection the ROS damage sustained by sperm can have negative consequences for early embryonic development and viability [69].

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Interestingly, our data also indicate that fructose (a monosaccharide) and sucrose (a disaccharide of fructose + glucose) have different effects on embryo mortality. In both A and B females, peak mortality was shifted in the direction of diets with increasing P when flies were fed on fructose-based diets. This pattern implies that the difference in embryo mortality was at the level of the relationship between the sugars to protein. Why this should be the case, however, is unknown, and deserves further study. Perhaps fructose and high P consumption had synergistic effects on ejaculate constituents through ROS damage [39,70].

Our analyses also revealed pronounced dietary effects on all three body nutrient reserves that we measured, providing a robust demonstration that dietary restriction had deteriorative effects on male body condition. Effects of macronutrient levels and/or interactions between them were significant for all body nutrients, being strongest for lipid and protein stores, and highly consistent with a previous study of *D. melanogaster* [35]. Crucially, we found that all of our individual measures of male body condition scaled negatively with embryo mortality among B females, and when condition was analysed as a mean value, the relationship was statistically significant. The caloric effect on embryo mortality, in contrast, was absent for A females, and predictably, there was no statistically significant relationship between male body condition and embryo mortality for A females either.

In sum, the results support the hypothesis that diet-induced variation in the metabolic state of sires influences early developmental processes in offspring and post-fertilization fitness outcomes. This link may have implications for understanding the consequences of suboptimal diet for ejaculate quality and reproductive fitness in animals generally, and for elucidating related processes in natural populations. Our data predict that when expression of male signalling traits is body condition-dependent [71], female mate choice on the basis of ornamentation may be selected as a function of a previously underappreciated and relatively 'cryptic' direct benefit in the form of a paternally driven influence on embryo viability.

Data accessibility. Datasets have been deposited in Dryad as http://dx.doi.org/10.5061/dryad.f35n7 [72].

Authors' contributions. M.P. designed and performed the experiments, analysed the data, and wrote the manuscript. L.W.S. designed and performed the experiments. J.B.B. performed physiological assays of nutrient reserves. K.R. and S.M.S.-B. contributed to data analyses and interpretation. S.J.S. designed the experiments and contributed to data interpretation.

Competing interests. The authors have no competing interests.

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References

1. Clutton-Brock TH. 1991 *The evolution of parental care*. Princeton, NJ: Princeton University Press.
2. Trivers RL. 1972 Parental investment and sexual selection. In *Sexual selection and the descent of man 1871–1971* (ed. B Campbell), pp. 136–179. Chicago, IL: Aldine.
3. Ayad A, Touati K, Iguer-Ouada M, Benbarek H. 2012 Different factors affecting the embryonic mortality in cattle. *Res. Opin. Anim. Vet. Sci.* **2**, 559–572.
4. Hemmings N, West M, Birkhead TR. 2012 Causes of hatching failure in endangered birds. *Biol. Lett.* **8**, 964–967. (doi:10.1098/rsbl.2012.0655)
5. Simmons LW, García-González F. 2007 Female crickets trade offspring viability for fecundity. *J. Evol. Biol.* **20**, 1617. (doi:10.1111/j.1420-9101.2007.01320.x)
6. Stockley P. 2003 Female multiple mating behaviour, early reproductive failure and litter size variation in mammals. *Proc. R. Soc. Lond. B* **270**, 271–278. (doi:10.1098/rspb.2002.2228)
7. Thayer LR, Hamlin HJ. 2015 Investigating factors contributing to reduced embryo survival in farm-raised Atlantic salmon, *Salmo salar* L. *Aquaculture Res.* **47**, 3980–3989. (doi:10.1111/are.12848)
8. Larsen EC, Christiansen OB, Kolte AM, Macklon N. 2013 New insights into mechanisms behind miscarriage. *BMC Med.* **11**, 154. (doi:10.1186/1741-7015-11-154)
9. Brewer CJ, Balen AH. 2010 The adverse effects of obesity on conception and implantation. *Reproduction* **140**, 347–364. (doi:10.1530/REP-09-0568)
10. Wu G, Bazer FW, Cudd TA, Meininger CJ, Spencer TE. 2004 Maternal nutrition and fetal development. *J. Nutr.* **9**, 2169–2172.
11. Wu G *et al.* 2013 Impacts of arginine nutrition on embryonic and fetal development in mammals. *Amino Acids* **45**, 241–256. (doi:10.1007/s00726-013-1515-z)
12. Bateman AJ. 1948 Intra-sexual selection in *Drosophila*. *Heredity* **2**, 349–368. (doi:10.1038/hdy.1948.21)
13. Dewsbury DA. 1982 Ejaculate cost and male choice. *Am. Nat.* **119**, 601–610. (doi:10.1086/283938)
14. Zeh DW, Smith RL. 1985 Paternal investment by terrestrial arthropods. *Am. Zool.* **25**, 785–805. (doi:10.1093/icb/25.3.785)
15. Poiani A. 2006 Complexity of seminal fluid: a review. *Behav. Ecol. Sociobiol.* **60**, 289–310. (doi:10.1007/s00265-006-0178-0)
16. McGraw LA, Suarez SS, Wolfner MF. 2015 On a matter of seminal importance. *Bioessays* **37**, 142–147. (doi:10.1002/bies.201400117)
17. Schjenken JE, Robertson SA. 2015 Seminal fluid signalling in the female reproductive tract: implications for reproductive success and offspring health. In *The male role in pregnancy loss and embryo implantation failure* (ed. R Bronson), pp. 127–158. Switzerland: Springer International Publishing.
18. Rando OJ. 2012 Daddy issues: paternal effects on phenotype. *Cell* **151**, 702–708. (doi:10.1016/j.cell.2012.10.020)
19. Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ, Robertson SA. 2014 Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proc. Natl Acad. Sci. USA* **111**, 2200–2205. (doi:10.1073/pnas.1305609111)
20. delBarco-Trillo J, García-Álvarez O, Soler AJ, Tourmente M, Garde JJ, Roldan ER. 2016 A cost for high levels of sperm competition in rodents: increased sperm DNA fragmentation. *Proc. R. Soc. B* **283**, 20152708. (doi:10.1098/rspb.2015.2708)
21. Kenagy GJ, Trombulak SC. 1986 Size and function of mammalian testes in relation to body size. *J. Mammal.* **67**, 1–22. (doi:10.2307/1380997)
22. Pitnick S. 1996 Investment in testes and the cost of making long sperm in *Drosophila*. *Am. Nat.* **148**, 57–80. (doi:10.1086/285911)
23. Solon-Biet SM *et al.* 2015 Macronutrient balance, reproductive function, and lifespan in aging mice. *Proc. Natl Acad. Sci. USA* **112**, 3481–3486. (doi:10.1073/pnas.1422041112)
24. Simmons LW. 2012 Resource allocation trade-off between sperm quality and immunity in the field

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cricket, *Teleogryllus oceanicus*. *Behav. Ecol.* **23**, 168–173. (doi:10.1093/beheco/arr170)

25. Van Voorhies WA. 1992 Production of sperm reduces nematode lifespan. *Nature* **360**, 456–458. (doi:10.1038/360456a0)
26. Bunning H, Rapkin J, Belcher L, Archer CR, Jensen K, Hunt J. 2015 Protein and carbohydrate intake influence sperm number and fertility in male cockroaches, but not sperm viability. *Proc. R. Soc. B* **282**, 20142144. (doi:10.1098/rspb.2014.2144)
27. Sbilordo SH, Grazer VM, Demont M, Martin OY. 2011 Impacts of starvation on male reproductive success in *Tribolium castaneum*. *Evol. Ecol. Res.* **13**, 347–359.
28. Stürup M, Baer-Imhoof B, Nash DR, Boomsma JJ, Baer B. 2013 When every sperm counts: factors affecting male fertility in the honeybee *Apis mellifera*. *Behav. Ecol.* **24**, 1192–1198. (doi:10.1093/beheco/art049)
29. Simpson SJ, Raubenheimer D. 2012 *The nature of nutrition: a unifying framework from animal adaptation to human obesity*. Princeton, NJ: Princeton University Press.
30. Morimoto J, Wigby S. 2016 Differential effects of male nutrient balance on pre-and post-copulatory traits, and consequences for female reproduction in *Drosophila melanogaster*. *Sci. Rep.* **6**, 27673. (doi:10.1038/srep27673)
31. Maklakov AA, Simpson SJ, Zajitschek F, Hall MD, Dessmann J, Clissold F, Raubenheimer D, Bondurianski R, Brooks RC. 2008 Sex-specific fitness effects of nutrient intake on reproduction and lifespan. *Curr. Biol.* **18**, 1062–1066. (doi:10.1016/j.cub.2008.06.059)
32. Fanson BG, Taylor PW. 2012 Protein : carbohydrate ratios explain life span patterns found in Queensland fruit fly on diets varying in yeast : sugar ratios. *Age* **34**, 1361–1368. (doi:10.1007/s11357-011-9308-3)
33. Jensen K, McClure C, Priest NK, Hunt J. 2015 Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in *Drosophila melanogaster*. *Aging Cell* **14**, 605–615. (doi:10.1111/acel.12333)
34. Lee KP, Simpson SJ, Clissold FJ, Brooks R, Ballard JWO, Taylor PW, Soran N, Raubenheimer D. 2008 Lifespan and reproduction in *Drosophila*: new insights from nutritional geometry. *Proc. Natl Acad. Sci. USA* **105**, 2498–2503. (doi:10.1073/pnas.0710787105)
35. Skorupa DA, Dervisefendic A, Zwiener J, Pletcher SD. 2008 Dietary composition specifies consumption, obesity, and lifespan in *Drosophila melanogaster*. *Aging Cell* **7**, 478–490. (10.1111/j.1474–9726.2008.00400.x)
36. Widdowson EM, McCance RA. 1935 The available carbohydrate of fruits: determination of glucose, fructose, sucrose and starch. *Biochem. J.* **29**, 151. (doi:10.1042/bj0290151)
37. Wrolstad RE. 2012 *Food carbohydrate chemistry*. Chichester, UK: John Wiley & Sons, Inc. and Institute of Food Technologists.
38. Lushchak OV, Gospodaryov DV, Rovenko BM, Yurkevych IS, Perkhulyn NV, Lushchak VI. 2013 Specific dietary carbohydrates differentially influence the life span and fecundity of *Drosophila melanogaster*. *J. Gerontol. Ser. A: Biomed. Sci. Med. Sci.* **69**, 3–12. (doi:10.1093/gerona/glt077)
39. Rovenko BM, Perkhulyn NV, Gospodaryov DV, Sanz A, Lushchak V, Lushchak VI. 2015 High consumption of fructose rather than glucose promotes a diet-induced obese phenotype in *Drosophila melanogaster*. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **180**, 75–85. (doi:10.1016/j.cbpa.2014.11.008)
40. Miyamoto T, Amrein H. 2014 Diverse roles for the *Drosophila* fructose sensor Gr43a. *Fly* **8**, 19–25. (doi:10.4161/fly.27241)
41. Ja WW, Carvalho GB, Zid BM, Mak EM, Brummel T, Benzer S. 2009 Water and nutrient-dependent effects of dietary restriction on *Drosophila* lifespan. *Proc. Natl Acad. Sci. USA* **106**, 18 633–18 637. (doi:10.1073/pnas.0908016106)
42. Food and Agricultural Organization WHO, United Nations University. 2004 Human energy requirements. *Food and Nutrition Technical Report*. Rome, 2001; FAO.
43. Broadie K, Skaer H, Bate M. 1992 Whole-embryo culture of *Drosophila*: development of embryonic tissues *in vitro*. *Dev. Genes Evol.* **201**, 364–375.
44. Campos-Ortega JA, Hartenstein V. 1997 *The embryonic development of* Drosophila melanogaster, 2nd edn. Berlin, Germany: Springer-Verlag.
45. Bownes M. 1975 A photographic study of development in the living embryo of *Drosophila melanogaster*. *Development* **33**, 789–801.
46. Berg JJ, Tymoczko JL, Stryer L. 2002 *Biochemistry*, 5th edn. New York, NY: W.H. Freeman.
47. Wood SN. 2006 *Generalized additive models: An introduction with R*. Boca Raton, FL: Chapman & Hall/CRC.
48. Wood S. 2016 *Package 'mgcv' Version 1.8–16*. The R Project for Statistical Computing.
49. Team RC. 2016 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
50. JMP®. Version 12.1.0 SAS Institute Inc., Cary, NC.
51. Bakos HW, Mitchell M, Setchell BP, Lane M. 2011 The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model. *Int. J. Androl.* **34**, 402–410. (doi:10.1111/j.1365-2605.2010.01092.x)
52. Kasturi SS, Tannir J, Brannigan RE. 2008 The metabolic syndrome and male infertility. *J. Androl.* **29**, 251–259. (doi:10.2164/jandrol.107.003731)
53. Linklater JR, Wertheim B, Wigby S, Chapman T. 2007 Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. *Evolution* **61**, 2027–2034. (doi:10.1111/j.1558-5646.2007.00157.x)
54. Sirot LK, Buehner NA, Fiumera AC, Wolfner MF. 2009 Seminal fluid protein depletion and replenishment in the fruit fly, *Drosophila melanogaster*: an ELISA-based method for tracking individual ejaculates. *Behav. Ecol. Sociobiol.* **63**, 1505–1513. (doi:10.1007/s00265-009-0806-6)
55. Sirot LK, Wolfner MF, Wigby S. 2011 Protein-specific manipulation of ejaculate composition in response to female mating status in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* **108**, 9922–9926. (doi:10.1073/pnas.1100905108)
56. Wigby S, Sirot LK, Linklater JR, Buehner N, Calboli FCF, Bretman A, Wolfner MF, Chapman T. 2009 Seminal fluid protein allocation and male reproductive success. *Curr. Biol.* **19**, 751–757. (doi:10.1016/j.cub.2009.03.036)
57. Reinhardt K, Naylor R, Siva-Jothy MT. 2011 Male mating rate is constrained by seminal fluid availability in bedbugs, *Cimex lectularius*. *PLoS ONE* **6**, e22082. (doi:10.1371/journal.pone.0022082)
58. Pyle DW, Gromko MH. 1978 Repeated mating by female *Drosophila melanogaster*: the adaptive importance. *Cell. Mol. Life Sci.* **34**, 449–450. (doi:10.1007/BF01935920)
59. Chapman T. 2001 Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity* **87**, 511–521. (doi:10.1046/j.1365-2540.2001.00961.x)
60. Simmons LW, Beveridge M. 2011 Seminal fluid affects sperm viability in a cricket. *PLoS ONE* **6**, e17975. (doi:10.1371/journal.pone.0017975)
61. Wolfner MF. 2002 The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in *Drosophila*. *Heredity* **88**, 85–93. (doi:10.1038/sj.hdy.6800017)
62. Gillott C. 2003 Male accessory gland secretions: modulators of female reproductive physiology and behavior. *Annu. Rev. Entomol.* **48**, 163–184. (doi:10.1146/annurev.ento.48.091801.112657)
63. Lung O, Kuo L, Wolfner MF. 2001 *Drosophila* males transfer antibacterial proteins from their accessory gland and ejaculatory duct to their mates. *J. Insect. Physiol.* **47**, 617–622. (doi:10.1016/S0022-1910(00)00151-7)
64. Samakovlis C, Kylsten P, Kimbrell DA, Engstrom A, Hultmark D. 1991 The andropin gene and its product, a male-specific antibacterial peptide in *Drosophila melanogaster*. *EMBO J.* **10**, 163.
65. Marchini D, Marri L, Rosetto M, Manetti AGO, Dallai R. 1997 Presence of antibacterial peptides on the laid egg chorion of the medfly *Ceratitis capitata*. *Biochem. Biophys. Res. Commun.* **240**, 657–663. (doi:10.1006/bbrc.1997.7694)
66. Rovenko BM, Kubrak OI, Gospodaryov DV, Perkhulyn NV, Yurkevych IS, Sanz A, Lushchak V, Lushchak VI. 2015 High sucrose consumption promotes obesity whereas its low consumption induces oxidative stress in *Drosophila melanogaster*. *J. Insect. Physiol.* **79**, 42–54. (doi:10.1016/j.jinsphys.2015.05.007)
67. Warbrick-Smith J, Behmer ST, Lee KP, Raubenheimer D, Simpson SJ. 2006 Evolving resistance to obesity in an insect. *Proc. Natl Acad. Sci. USA* **103**, 14 045–14 049. (doi:10.1073/pnas.0605225103)

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68. Dowling DK, Simmons LW. 2009 Reactive oxygen species as universal constraints in life-history evolution. *Proc. R. Soc. B* **276**, 1737–1745. (doi:10.1098/rspb.2008.1791)
69. Chen H, Liao S.-B., Cheung MPL, Chow PH, Cheung ALM. 2012 Effects of sperm DNA damage on the levels of RAD51 and p53 proteins in zygotes and 2-cell embryos sired by golden hamsters without the major accessory sex glands. *Free Radical Biol. Med.* **53**, 885–892. (doi:10.1016/j.freeradbiomed.2012.06.007)
70. Lushchak V, Rovenko BM, Gospodaryov DV, Lushchak VI. 2011 *Drosophila melanogaster* larvae fed by glucose and fructose demonstrate difference in oxidative stress markers and antioxidant enzymes of adult flies. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **160**, 27–34. (doi:10.1016/j.cbpa.2011.04.019)
71. Tomkins JL, Radwan J, Kotiaho JS, Tregenza T. 2004 Genetic capture and resolving the lek paradox. *Trends Ecol. Evol.* **19**, 323–328. (doi:10.1016/j.tree.2004.03.029)
72. Polak M, Simmons LW, Benoit JB, Ruohonen K, Simpson SJ, Solon-Biet SM. 2017 Data from: Nutritional geometry of paternal effects on embryo mortality. Dryad Digital Repository. (http://dx.doi.org/10.5061/dryad.f35n7)