JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.13384

Developmental diet irreversibly shapes male post-copulatory traits in the neriid fly *Telostylinus angusticollis*

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Keywords:

condition dependence;
diet;
dietary restriction;
plasticity;
post-copulatory;
reproduction;
sperm;
in vivo sperm movement.

Abstract

Nutrient availability has been shown to influence investment in many fitness-related traits, including male reproductive success. Many studies have demonstrated that a reduction in nutrient availability alters male post-copulatory trait expression, with some studies demonstrating an effect of developmental nutrients and others, an effect of adult nutrients. However, few studies have manipulated both developmental and adult nutrients in the same experiment. Therefore, it is not clear what life-stage has the greatest effect on post-copulatory trait expression, and if the effects of developmental and adult nutrients can interact. Here, we investigate effects of developmental and adult nutrition on male testes and accessory gland size, sperm movement within the female reproductive tract and sperm length in the neriid fly, *Telostylinus angusticollis*. We found that males fed a nutrient-poor developmental diet produced sperm with a reduced tail beat frequency and had smaller testes and accessory glands compared to males fed a nutrient-rich developmental diet. In contrast, we found no effects of adult nutrition on any traits measured, although sperm length was correlated with body size and male age but unaffected by nutrition at any stage. Therefore, investment in adult post-copulatory traits is determined early on by developmental nutrients in male neriid flies, and this effect is not altered by adult nutrient availability.

Introduction

Male post-copulatory traits, such as sperm and semen traits, can be highly susceptible to changes in the male environment (reviewed in Reinhardt *et al.*, 2015). For example, a reduction in nutrient availability often results in a decrease in post-copulatory trait expression (e.g. Droney, 1998; Kahrl & Cox, 2015; Kaldun & Otti, 2016; Wigby *et al.*, 2016; Duplouy *et al.*, 2017), likely due to condition-dependent investment whereby males that have fewer metabolic resources (low condition individuals) are less able to invest in costly life-history traits (Andersson, 1982; Nur & Hasson, 1984; Rowe & Houle, 1996). Yet, some studies show that low condition males invest more in post-copulatory traits (e.g. Perry & Rowe, 2010; Mehlis *et al.*, 2015), perhaps because low condition males tend to have reduced mating success, and may therefore strategically invest their limited resources into sperm and ejaculate traits to make the most of any mating opportunities that arise (Parker, 1990; Williams *et al.*, 2005; Cameron *et al.*, 2007; Parker & Pizzari, 2010). These differences among studies likely reflect species differences in reproductive ecology (Simmons *et al.*, 1999; Cornwallis & Birkhead, 2008; Reinhardt *et al.*, 2015). However, another factor that is often overlooked is the timing of nutritional

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restriction. Environmental impacts experienced during developmental stages may have very different consequences to those experienced during adult stages.

Most studies that have examined the effects of diet on post-copulatory traits have manipulated the adult diet. For example, Droney (1998) demonstrated that reduced protein in the adult diet of *Drosophila grimshawi* significantly reduced testes and accessory gland size, and Evans *et al.* (2015) demonstrated that reduced food quantity in adult male guppies (*Poecilia reticulata*) reduced sperm number, sperm viability and sperm velocity. In contrast, Perry & Rowe (2010) demonstrated that male ladybirds (*Adalia bipunctata)* fed a reduced quantity of food produced smaller ejaculates, but these ejaculates contained a higher total number of sperm, suggesting that males were investing more in the sperm component of the ejaculate and less in the nonsperm components such as proteins in the semen. Hence, nutrient restriction at the adult stage can have both positive and negative effects on male post-copulatory traits.

Early-life conditions can also have long-lasting effects on individual fitness (Barker, 2004), and a number of studies have shown that nutrient-restricted juvenile males have reduced post-copulatory trait expression compared to fully fed males ('silver spoon' hypothesis) (Qvarnstrom & Price, 2001). For example, Vega-Trejo *et al.* (2016) demonstrated that male mosquito fish (*Gambusia holbrooki*) fed a restricted juvenile diet had a much lower rate of sperm replenishment compared to fully fed males, and Dávila & Aron (2017) demonstrated that male ant larvae (*Linepithema humile*) deprived of nutrients produced fewer sperm. However, Mehlis *et al.* (2015) demonstrated that male three-spined sticklebacks (*Gasterosteus aculeatus*) raised under food-restricted conditions outcompeted fully fed males in sperm competition. Hence, nutrient restriction during developmental stages can also positively or negatively affect male post-copulatory trait expression. However, few studies have manipulated nutrient availability during both developmental and adult stages in the same experiment (although see Amitin & Pitnick, 2007; Vermeulen *et al.*, 2008; Melo *et al.*, 2014). Therefore, the relative importance of nutrient availability in each life-history stage for male post-copulatory investment, and potential for interactive effects of juvenile and adult nutrition, remains poorly understood.

The effects of developmental and adult nutrients cannot be fully understood in isolation as nutrient availability at both life-stages could have additive and interactive effects on male investment in post-copulatory traits. For example, poor nutrition during development can alter how nutrients are mobilized in adults (Gheorghe *et al.*, 2010). In some cases, fitness may be optimized when adult and developmental environmental conditions match, because the juvenile environment can predispose individuals to function well under similar environmental conditions as adults (DeWitt *et al.*, 1998; Monaghan, 2008). Alternatively, individuals that have a nutrient-poor developmental environment may be able to compensate for this deprivation when provided with plentiful resources as adults (Mevi-Schütz & Erhardt, 2005; Müller & Müller, 2016), and this could also result in a cumulative effect of nutrient deprivation for individuals that are deprived of nutrients at both life-stages.

Here, we examine both additive and interactive effects of developmental and adult nutrient restriction on male post-copulatory trait expression in the neriid fly, *Telostylinus angusticollis*. Male neriids raised on a nutrient-rich developmental diet are larger and show increased investment in precopulatory reproductive traits (Bonduriansky, 2007; Sentinella *et al.*, 2013; Hooper *et al.*, 2017). Male developmental diet also influences offspring viability and growth (Bonduriansky, 2007; Crean *et al.*, 2014; Runagall-McNaull *et al.*, 2015), and male developmental and adult diets can interact to influence egg hatching success (Macartney *et al.*, 2017). It is not known how nutrient availability influences investment in post-copulatory traits in this species, but post-copulatory trait expression is likely to be important because females are polyandrous and males therefore face sperm competition. We predicted that a nutrient-poor developmental diet would have a strong negative effect on testes and accessory gland size as these are expected to co-vary with body size (Hellriegel & Blanckenhorn, 2002). There is also evidence that adult diet can negatively affect testes and accessory gland size in other Dipteran species (Droney, 1998; Baker *et al.*, 2003), so we asked whether this is the case in *T. angusticollis*. Sperm traits can also be influenced by nutrient limitation either during development (Hellriegel & Blanckenhorn, 2002; Avila *et al.*, 2011; Tomášek *et al.*, 2017) or during the adult stage (Rahman *et al.*, 2013; Evans *et al.*, 2015; Kahrl & Cox, 2015), so we asked how sperm movement and sperm size are affected by nutrition, which life-stage has the strongest effect, and whether juvenile and adult nutrition have interactive effects on these traits.

Materials and methods

Experimental set-up

Eggs were collected from stock cages of *T. angusticollis*, originally sourced from multiple locations around Sydney, Australia, and maintained as a large outbred population. Eggs were transferred in alternating order onto 150 g of 'nutrient-rich' ('R') or 'nutrient-poor' ('P') developmental (i.e. larval) diet (50 eggs per container, 20 replicate containers per treatment). The nutrient-poor developmental diet was manipulated by reducing the total nutrient concentration (both protein and

carbohydrates) by three-fold. The rich developmental diet consisted of 30 mL of sugar cane molasses (Conga Foods Pty. Ltd, Preston, Vic., Australia), 30 mL of liquid barley malt (Colonial Farms brand, Select Foods Pty. Ltd., Smithfield, NSW, Australia) and 32 g of Soya protein powder (Nature's Way brand; Pharm-a-care Pty. Ltd., Warriewood, NSW, Australia) per litre of cocopeat (the substrate for the larvae to forage in) hydrated with 600 mL of water. The poor developmental diet consisted of 10 mL of sugar cane molasses, 10 mL of liquid barley malt and 10 g of Soya protein powder per litre of cocopeat hydrated with 600 mL of water. This manipulation of the developmental diet has been used extensively in our laboratory and has been shown to have wide-ranging implications for male phenotype and performance (e.g. Bonduriansky, 2007; Hooper *et al.*, 2017; Macartney *et al.*, 2017). Developmental containers were kept at 25 °C with a 12-h to 12-h light–dark cycle and watered periodically.

On the day of adult emergence, males from each developmental diet were randomly assigned to either a 'nutrient-rich' ('R') adult diet of brown sugar and yeast, or a 'nutrient-poor' ('P') adult diet of brown sugar only, in a fully crossed design with four diet combinations (RR, RP, PR, PP) where flies had *ad libitum* access to the developmental and adult diet provided. This adult dietary manipulation (which limits protein but not carbohydrates) differs from the developmental manipulation (which limits both protein and carbohydrates) because *T. angusticollis* has substantially different nutrient requirements in developmental vs. adult stages. Developing larvae require a relatively balanced mixture of protein and carbohydrates (Sentinella *et al.*, 2013; Runagall-McNaull *et al.*, 2015). By contrast, adult males require carbohydrates to survive, but can survive and reproduce with minimal dietary protein (Adler *et al.*, 2013; Macartney *et al.*, 2017). Nonetheless, protein is a key nutrient for male reproduction in many species (Droney, 1998; Baker *et al.*, 2003; Costa *et al.*, 2012), and we have previously found context-dependent effects of adult dietary protein on male reproductive performance in *T. angusticollis* (Adler *et al.*, 2013; Macartney *et al.*, 2017). Males were housed with five other males per cage to promote investment in post-copulatory traits (but only one male from each cage was included in the study). They were not housed with any females in order to ensure the males remained virgins before the sperm movement assay. The mean age of experimental males was 23.3 ± 9.64 (SD) days at the time of the assay. This is a relatively young age as male neriid flies can live up to 150 days in the laboratory (Hooper *et al.*, 2017). Cages were lined with cocopeat and watered daily to provide hydration. Two separate blocks of the experiment were completed (block 1: $N = 51$; block 2: $N = 52$, with $n = 11–15$ individuals/treatment combination/block).

Sperm movement within the female reproductive tract

Individual males were paired with a virgin female (all reared on a rich developmental diet and standardized for age) and observed until mating occurred. Females were then killed by crushing their thorax, and their reproductive tract was dissected into a drop of phosphate-buffered saline solution. Not all males transferred sperm on this initial mating, but the probability of sperm transfer was not influenced by treatment (supplementary material).

Each individual sperm as well as all sperm collectively move in an undulating, spiral motion. Sperm also move collectively in a spherical motion within the spermathecae (Video S1). Because of this, traditional CASA approaches are not a viable method for tracking sperm movement. Instead, we used a new technique that allows us to measure the tail beat frequency to quantify sperm movement by using Fourier analysis on intensity vs. time traces extracted on a pixel-by-pixel basis from the time-stamped image stacks using a custom-written GUI in MATLAB (see supplementary material and Nicovich *et al.*, 2015 for details). This measure is likely to be more functionally relevant for sperm performance during sperm competition than *in vitro* measures of sperm velocity, as the sperm are moving in a natural environment rather than an artificial substrate (Curtis & Benner, 1991; Werner *et al.*, 2007; Lüpold & Pitnick, 2018).

Male Morphological measurements

Flies were photographed at 6.5× magnification to measure thorax length as a measure of body size (Bonduriansky, 2007), and testes and three types of accessory glands ('epitesticular', 'lobate' and 'tubular' (Fig. S1)) were dissected on to a slide moistened with saline solution and photographed at 25× magnification. Testes were then severed to release sperm and a coverslip placed over the sample. Five to eight sperm per male were photographed at 400× magnification and a mean taken. Thorax length, testes and accessory glands were photographed using a Zeiss AxioCamERc5s camera mounted to a Zeiss Stemi 2000-CS microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA), and sperm were photographed using a Zeiss AxioCam HSc camera mounted on a Zeiss AxioScope A1 compound microscope. Thorax length, testes area, accessory gland area and sperm length measurements were obtained from images using ImageJ software (Rasband, 2015). Each trait was measured twice, and the average value was used in analyses to reduce measurement error.

Statistical analysis

All analyses were completed in R version 3.3.2, using the lme4 (Bates *et al.*, 2015) and LmerTest (Kuznetsova *et al.*, 2017) packages. Linear mixed effects models (Gaussian distributions) were used to test main effects and interactions of developmental and adult diets on all male post-copulatory traits with block as a random factor. LmerTest calculates *P*-values and degrees of freedom for Gaussian mixed models based on the Satterthwaite approximation for denominator degrees of freedom (Schaalje *et al.*, 2002). Thorax length was centred within developmental diet treatment groups because the developmental diet affects thorax length, making the diet treatment partially redundant with thorax length, and centred thorax length was included as a co-variate in the models to test for correlations of relative body size and trait size within developmental diets. Male age was initially included as a co-variate in all post-copulatory trait models but was later removed from the models, except sperm length, as its inclusion did not significantly improve model fit (based on likelihood ratio tests (LRTs) with a conservative cut-off of $P > 0.1$) (Table S1 for LRTs). Sperm movement was analysed with two large outliers removed (both males reared on a nutrient-rich developmental diet with sperm movement > four standard deviations above the mean). Removal of these outliers makes our results more conservative. Sperm length was analysed with one outlier removed, and the epitesticular gland was analysed with one outlier removed. Outliers were identified as being greater than four standard deviations away from the mean.

Validation of all models was conducted using visual assessment of residuals. Values in results are reported as mean ± SE.

Results

The nutrient-poor developmental diet significantly reduced male thorax length (rich developmental diet: 2.90 ± 0.01 mm; poor developmental diet: 1.92 ± 0.03 mm; linear mixed effects model $t_{96} = 29.265$, $P < 0.001$). The nutrient-poor developmental diet also reduced testes size, accessory gland size and sperm movement within the female reproductive tract (Tables 1 and S2; Figs 1 and 2).

Sperm length was not affected by any of the diet manipulations (Table 1). However, sperm length was positively correlated with thorax length (centred within developmental diet) (Fig. 3a) and was negatively correlated with male age (Table 1). Testes size was also positively correlated with centred thorax length (Fig. 3b), but there was no correlation between the size of any of the accessory glands and centred thorax length (Table 1).

Adult diet did not affect male thorax length (rich adult diet: 2.44 ± 0.07 mm; poor adult diet: 2.42 ± 0.07 mm; linear mixed effects model $t_{96} = 1.651$, $P = 0.102$). Adult diet also had no effect on any of the post-copulatory traits (Table 1). There was no significant interaction effect of developmental diet and adult diet on any of the traits measured (Table 1).

Discussion

We found that developmental nutrient availability has a long-lasting and perhaps irreversible effect on post-copulatory reproductive investment in male neriid flies. Males reared on a nutrient-poor developmental diet were not only smaller, they also had smaller testes and accessory glands, and produced slower sperm, regardless of the quality of the diet they were fed as adults. Testes size and sperm length were

Table 1 Effects of developmental and adult diet on male post-copulatory traits, with male thorax length (centred within developmental diets) as a co-variate for all response variables, and male age as a co-variate for sperm length. Bold values indicate a significance value of $P < 0.05$, and a positive effect indicates an increase in trait expression with a rich diet, with larger thorax length, or with age. Output from linear mixed-effect models with Gaussian distributions.

	Sperm movement		Testes size		Sperm length		Epitesticular accessory gland		Lobate accessory gland		Tubular accessory gland	
	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value
Intercept	**2.839**	**0.099**	**2.399**	**< 0.001**	**639.722**	**0.002**	**0.660**	**0.0506**	**1.258**	**< 0.001**	**2.076**	**< 0.001**
Developmental diet	**0.253**	**0.005**	**3.380**	**< 0.001**	−1.359	0.681	**1.667**	**< 0.001**	**0.728**	**< 0.001**	**0.982**	**< 0.001**
Adult diet	−0.038	0.666	0.138	0.509	1.525	0.657	0.061	0.804	0.059	0.625	0.009	0.969
Centred thorax length	0.176	0.411	**1.177**	**0.024**	**3.108**	**0.002**	0.120	0.804	0.525	0.059	0.476	0.415
Age	–	–	–	–	**−0.593**	**0.001**	–	–	–	–	–	–
Developmental diet × adult diet	0.050	0.688	−0.320	0.267	5.185	0.269	−0.292	0.280	0.064	0.685	−0.237	0.491

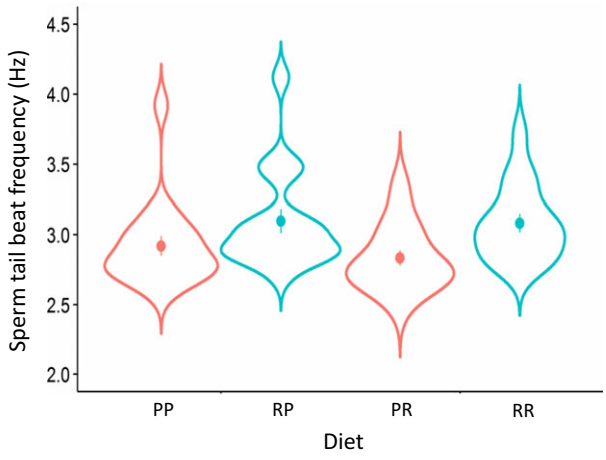

Fig. 1 Violin plots showing dietary effects on sperm tail beat frequency. Mean ± SE on inside of each plot. Red = poor developmental diet, blue = rich developmental diet.

positively correlated with thorax length centred within developmental diet treatment groups, suggesting that these traits are genetically correlated with body size (or potentially another environmental factor that is correlated with body size and was not controlled for in our study). Sperm size was also negatively associated with male age (in the narrow range of ages included in this study) yet was not affected by developmental or adult diet manipulations, indicating that sperm size does not respond plastically to dietary nutrients. Surprisingly, there was no effect of adult diet, nor an interactive effect of developmental and adult diet on any of the post-copulatory traits measured. This suggests that there is no compensatory or cumulative effect of adult diet and developmental diet, nor is there a benefit of environmental matching between developmental and adult diets in male neriid flies. Therefore, male post-copulatory traits appear to be most sensitive to developmental nutrients in

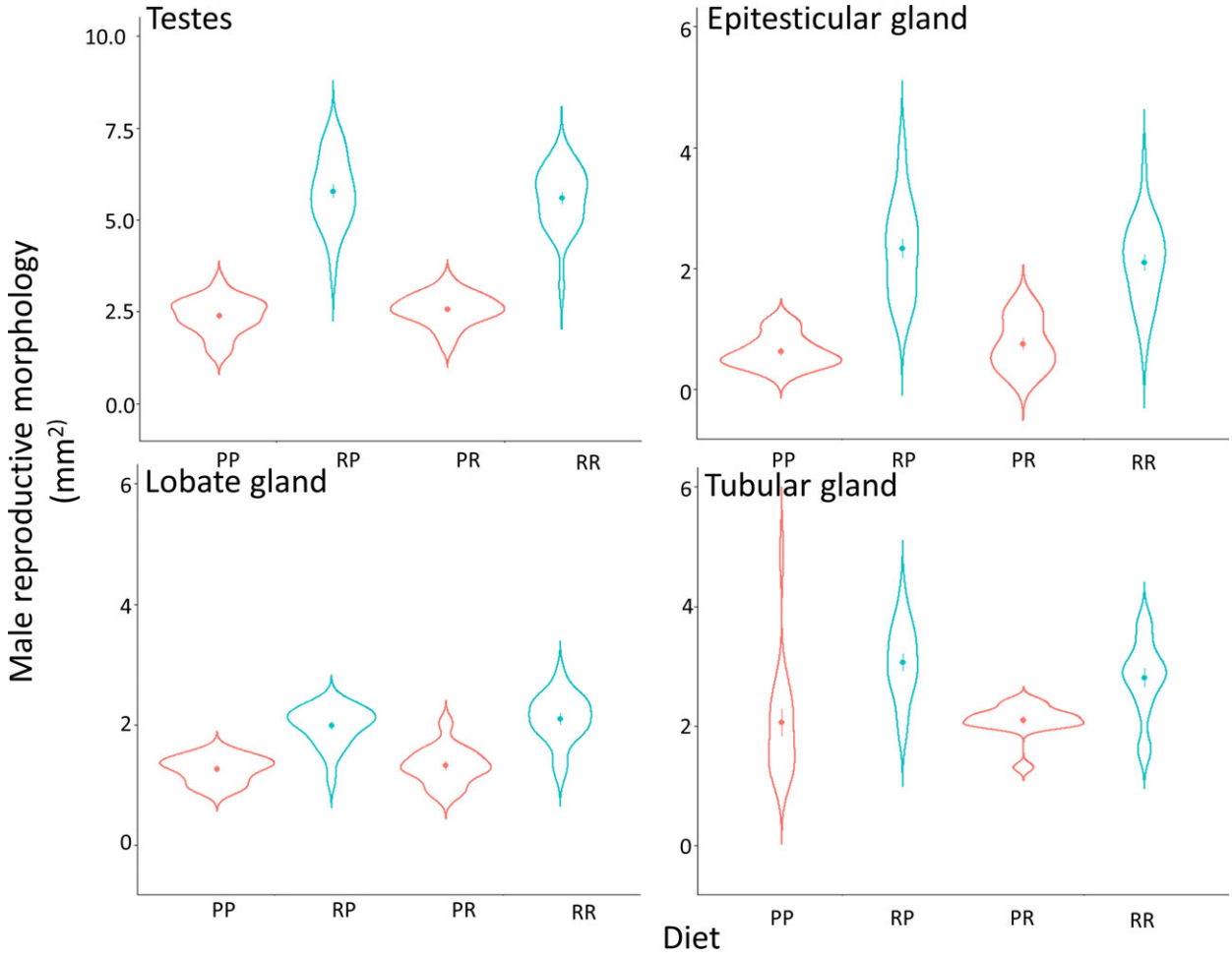

Fig. 2 Violin plots showing dietary effects on male reproductive morphology. Mean ± SE on inside of each plot. Red = poor developmental diet, blue = rich developmental diet.

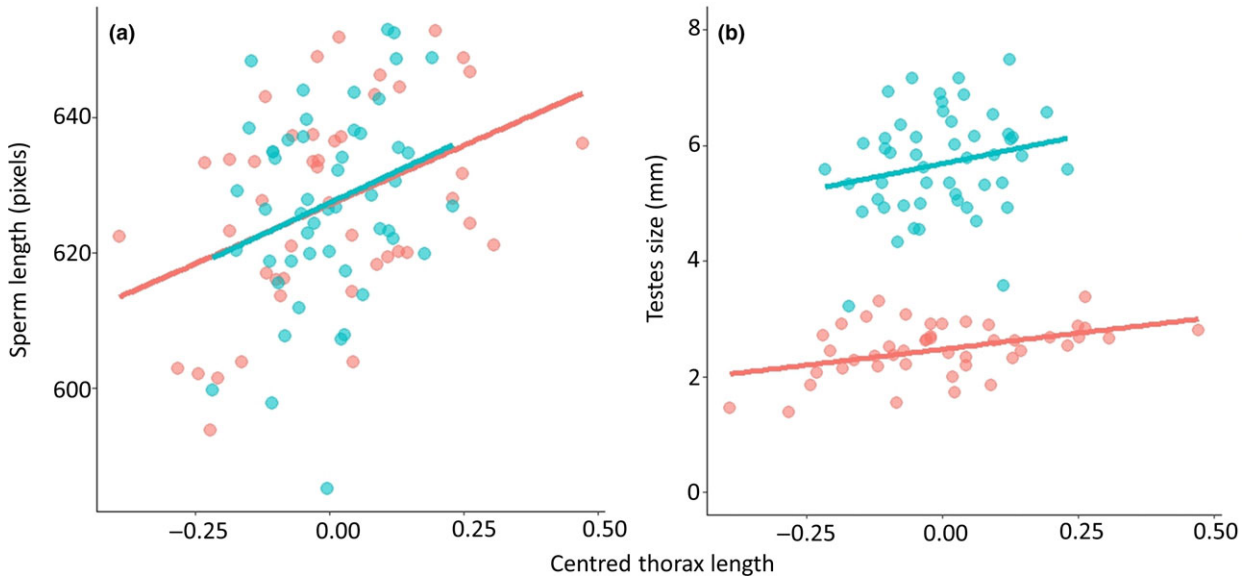

Fig. 3 Relationship between thorax length (centred within developmental diet) and sperm length (a), and testes size (b), grouped by developmental diet treatment. Red dots and line = poor developmental diet, blue dots and line = rich developmental diet.

T. angusticollis, with a nutrient-rich developmental diet providing a 'silver spoon' for post-copulatory trait expression.

Similar to our findings, male spiders (*Paratrechalea ornata*) subject to early food deprivation were unable to recover the quality of saliva produced nuptial gifts when provided with increased food later in life (Macedo-Rego *et al.*, 2016). However, in rats, although sperm production was reduced by *in utero* dietary restriction, sperm production was recovered when males were provided with a normal protein diet after weaning (Melo *et al.*, 2014). These differences between species may reflect differences in ontogeny of post-copulatory traits. For example, in holometabolous insects like neriid flies, the larval (developmental) stage is the phase where nutrients are allocated to body growth and extra metabolites are stored for use in the adult stage (Boggs, 1981). Allocation to imaginal discs that develop into adult reproductive structures like testes and accessory glands also occurs during the developmental stage. Nutrition during development may also shape epigenetic programming of imaginal disc tissues that develop into the male germ line, with lifelong effects on gene-expression patterns in the testes (and potentially sperm, affecting sperm movement) and accessory glands. However, it is interesting that other holometabolous insects can compensate for a scarcity of developmental resources in other fitness-enhancing traits (Mevi-Schütz & Erhardt, 2005; Müller & Müller, 2016), suggesting that regulation of post-copulatory trait expression can vary substantially between species.

The positive effects of developmental nutrient availability on post-copulatory trait expression in male *T. angusticollis* are consistent with other studies on this species that have found positive effects of developmental nutrient availability on fitness-related precopulatory traits. Males reared on a nutrient-rich developmental diet have exaggerated secondary sexual morphologies (Bonduriansky, 2007), display increased territorial fighting behaviour for access to females (Hooper *et al.*, 2017) and gain matings faster than males reared on a nutrient-poor diet (Fricke *et al.*, 2015). We suspected that males reared on a nutrient-poor developmental diet may preferentially invest in post-copulatory traits to compensate for their reduced mating success and increased risk of sperm competition (Parker, 1990; Williams *et al.*, 2005; Cameron *et al.*, 2007; Parker & Pizzari, 2010). Indeed, *T. angusticollis* males reared on a nutrient-poor diet mate for longer when they do get the opportunity to mate (Fricke *et al.*, 2015). However, male *T. angusticollis* appear to be investing more in both pre- and post-copulatory traits when developmental nutrients are plentiful.

Restricting protein in the adult diet increases lifespan of both male and female neriid flies by 65% but renders females completely infertile (Adler *et al.*, 2013). Yet, despite these dramatic effects on lifespan and female reproduction, we found no effect of adult dietary protein restriction on male post-copulatory traits. Similarly, previous studies of *T. angusticollis* have only found subtle, context-dependent effects of adult diet on male reproduction (Adler *et al.*, 2013; Macartney *et al.*, 2017). Protein is a key nutrient for male reproduction

in other species (Droney, 1998; Baker *et al.*, 2003; Costa *et al.*, 2012), and it is therefore surprising that it has little influence on male reproduction in *T. angusticollis*. Male and female neriid flies require different quantities of developmental nutrients for optimal reproductive investment (Bonduriansky *et al.*, 2016), and this appears to equally apply to adult nutrient requirements. It is possible that restricting sugar in the adult diet may influence male reproductive traits. However, because *T. angusticollis* adults feed on tree sap, sugar is unlikely to be limiting in their natural diet, and substantial carbohydrate restriction causes rapid death (Adler *et al.*, 2013).

The reduction in testes and accessory gland size, and reduced sperm tail beat frequency observed in males reared on a nutrient deficient developmental diet is likely to impact male fitness through a reduction in fertilization success, particularly under sperm competition. A reduction in testes size due to developmental nutrient restriction is likely to reduce sperm production and replenishment rates (Schärer *et al.*, 2004), and thus reduce siring success (Vellnow *et al.*, 2018). A decrease in accessory gland size in males reared on a nutrient-restricted developmental diet is likely to reduce the production and/or storage capacity of accessory gland products (Linklater *et al.*, 2007), which can also affect male fitness (Perry *et al.*, 2013), particularly under high sperm competition risk (Bartlett *et al.*, 2017). Finally, higher sperm velocity has been shown to increase paternity share under sperm competition (Boschetto *et al.*, 2011; but see Lupold *et al.*, 2012). In neriid flies, sperm tail beat frequency probably determines the rate of sperm movement into the spermathecae (Nicovich *et al.*, 2015) and could affect male paternity share when faced with sperm competition between rival males. Therefore, males reared on the nutrient-poor developmental diet not only have reduced expression of pre-copulatory traits (e.g. Bonduriansky, 2007; Hooper *et al.*, 2017) which is likely to affect mating outcomes and increase the risk of sperm competition; they may also have reduced fertilization success when faced with sperm competition due to the reduction in testes size, accessory gland size and sperm movement within the female reproductive tract. Thus, a nutrient-poor developmental diet is likely to have significant consequences for male fitness and this is unlikely to be compensated for by plentiful adult nutrients.

Conclusions

Overall, there is no evidence that male testes and accessory gland size, sperm movement or sperm length are affected by an interaction between developmental and adult nutrition in *T. angusticollis*, nor are male post-copulatory traits affected by adult nutrition. Instead, we show that sperm movement, testes size and accessory gland size are most sensitive to developmental nutrition and respond in a condition-dependent manner. A nutrient-rich developmental diet provides a 'silver spoon' for adult male post-copulatory trait expression, whereas males fed a nutrient-poor developmental diet are less able to invest in post-copulatory traits even if they encounter abundant nutrients as adults. This irreversible reduction in male post-copulatory trait expression may then reduce male fitness under sperm competition.

Acknowledgments

We would like to thank the reviewers for their very thorough and insightful comments on this manuscript. Funding was provided by the Australian Research Council through a Discovery Early Career Researcher Award to AJC, and a Future Fellowship to RB. We have no conflict of interests to declare.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Putative accessory glands and testis (a, b) and internal reproductive structures of *Telostylinus angusticollis* (c).

Appendix S1 Supplementary material

Table S1 LRTs comparing the inclusion of male age as a co-variate in the models of male post-copulatory traits vs. removing male age from the models.

Table S2 Means and standard errors for each response variable in each combination of developmental and adult diet.

Video S1 Sperm moving within the sperm duct and spermathecae of a female neriid fly (*Telostylinus angusticollis*).

Data deposited at Dryad: https://doi.org/10.5061/dryad.0215pq7

Received 23 May 2018; revised 20 August 2018; accepted 23 September 2018