JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.13087

Adult dietary protein has age- and context-dependent effects on male post-copulatory performance

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Keywords:

age;
condition;
diet restriction;
males;
plasticity;
post-copulatory performance;
protein;
reproduction.

Abstract

The highly conserved effect of dietary protein restriction on lifespan and ageing is observed in both sexes and across a vast range of taxa. This extension of lifespan is frequently accompanied by a reduction in female fecundity, and it has been hypothesized that individuals may reallocate resources away from reproduction and into somatic maintenance. However, effects of dietary protein restriction on male reproduction are less consistent, suggesting that these effects may depend on other environmental parameters. Using the neriid fly, *Telostylinus angusticollis*, we examined age-specific effects of adult dietary protein restriction on male post-copulatory reproductive performance (fecundity and offspring viability). To explore the context dependence of these effects, we simultaneously manipulated male larval diet and adult mating history. We found that protein-restricted males sired less viable offspring at young ages, but offspring viability increased with paternal age and eventually exceeded that of fully fed males. The number of eggs laid by females was not affected by male dietary protein, whereas egg hatching success was subject to a complex interaction of male adult diet, age, larval diet and mating history. These findings suggest that effects of protein restriction on male reproduction are highly context dependent and cannot be explained by a simple reallocation of resources from reproduction to somatic maintenance. Rather, these effects appear to involve changes in the scheduling of male reproductive investment with age.

Introduction

A reduction in adult dietary nutrients has been shown to extend lifespan across a range of taxa extending from yeast to mammals (reviewed in Merry, 1995; Masoro, 2005; Partridge & Brand, 2005). Often, this extension of lifespan is accompanied by a reduction in fecundity, but this trade-off has predominantly been demonstrated in females (Partridge *et al.*, 2005; Lee *et al.*, 2008; Maklakov *et al.*, 2008; Adler *et al.*, 2013). It has been suggested that this reduction in fecundity results from a reallocation of energetic resources away from reproduction and into somatic maintenance, in order to increase the chance of outliving the period of nutrient scarcity and reproduce at a later date (Holliday, 1989; Shanley & Kirkwood, 2000; Davison *et al.*, 2014). However, although nutrient restriction has also been shown to increase the lifespan of males (Magwere *et al.*, 2004; Adler *et al.*, 2013), the effects on male fecundity have been less consistent (Fricke *et al.*, 2008; Gosden & Chenoweth, 2011; Zajitschek *et al.*, 2012; Adler *et al.*, 2013; Moatt *et al.*, 2016).

The lifespan extension and fecundity reduction effects were previously thought to be due to overall caloric restriction. However, it has been shown that these effects result primarily from protein restriction (Partridge *et al.*, 2005; Lee *et al.*, 2008; Solon-Biet *et al.*, 2015; Le Couteur *et al.*, 2016). Protein is a key macronutrient needed to produce ovules, and protein-restricted females may be unable to produce ovules when protein is restricted due to the costly nature of oogenesis (Chippindale *et al.*, 1993; Adler *et al.*, 2013). In contrast, the cost of producing sperm is thought to

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be much lower (Jonsson & Jonsson, 1997; Hayward & Gillooly, 2011), and therefore, males may be able to continue to produce an adequate supply of sperm even when protein is scarce. However, males cannot ensure reproductive success simply by producing sperm because fertilization success typically depends on the outcome of both pre- and post-copulatory competition. Hence, the lack of clear evidence for a reduction in male reproduction when dietary protein is restricted may be due to the complexity of measuring male investment into reproduction.

One way that males can increase their reproductive success is by investing in nonsperm components of the seminal fluid. The male ejaculate contains many components such as proteins, peptides and noncoding RNAs that can enhance multiple aspects of male fitness (Poiani, 2006; Perry *et al.*, 2013; Crean *et al.*, 2016). For example, seminal fluid proteins can be absorbed either into the female soma or directly into the ovules and can result in increased egg laying rate and increased offspring viability (Chapman, 2001; Wolfner, 2002; Sirot *et al.*, 2009; Wigby *et al.*, 2009; Avila *et al.*, 2011), and epigenetic factors such as noncoding RNAs can influence embryonic gene expression and offspring development (Eaton *et al.*, 2015; Crean *et al.*, 2016). Therefore, the cost of reproduction for males is not solely the production of sperm, but may involve other fecundity-enhancing traits. These costs are evident in species that invest in large spermatophores or nuptial gifts (Halliday & Houston, 1978; Linklater *et al.*, 2007; Perry *et al.*, 2013), but may also be important in species that transfer small ejaculates (Crean *et al.*, 2016). Protein restriction may limit the production of these nonsperm ejaculate components. Thus, protein restriction could manifest not only in reduced sperm quantity and quality but also in reduced ability to induce female oviposition or enhance offspring viability.

Nutrients acquired at different life stages are thought to be allocated to different traits, particularly in holometabolous insects (Boggs, 1981, 2009). For example, males reared in nutrient-poor conditions may invest less in secondary sexual characters (Emlen, 1997; Devigili *et al.*, 2013; Sentinella *et al.*, 2013). Such early allocation decisions could have lifelong consequences for adult reproductive strategies, and the effects of nutrient restriction in development may interact with protein restriction at the adult stage (Boggs, 2009). Social environment is another potentially important factor. In particular, frequent mating can deplete components of the ejaculate and/or reduce future reproductive effort (Halliday & Houston, 1978; Sirot *et al.*, 2009; Reinhardt *et al.*, 2011), so mating history is also likely to interact with the nutritional environment to influence male reproductive performance (Boggs, 2009; Zajitschek *et al.*, 2012). Moreover, because dietary protein influences the rate of ageing, these effects may be expected to manifest in an age-dependent manner (Masoro, 2005). If interactions with such factors are important, they could make the effects of protein on male reproductive performance more difficult to detect.

Here, we investigate age-dependent and context-dependent effects of protein restriction on male post-copulatory reproductive performance in the neriid fly *Telostylinus angusticollis*. In this species, the lifespan extension effect of adult dietary protein restriction has been demonstrated previously in both sexes but, whereas protein restriction rendered females completely infertile, costs to males were far less pronounced (Adler *et al.*, 2013). *Telostylinus angusticollis* males transfer a small ejaculate that is not packaged in a spermatophore and does not appear to contain a nutritive nuptial gift. However, multiple aspects of male fecundity are condition dependent, suggesting differential allocation of energetic resources to different aspects of the ejaculate depending on male environment (Adler & Bonduriansky, 2013; Adler *et al.*, 2013). We manipulated protein availability by providing adult males with both sugar and protein (fully fed diet), or sugar only (protein-restricted diet). To investigate the context dependence of the protein restriction effect, we simultaneously manipulated male developmental resources (which determine early-life condition) by varying total nutrient quantity in the larval diet and we manipulated male mating history allowing some males to mate throughout their life (*ad libitum* mating) while maintaining others as virgins until the fecundity assay (restricted mating) in a fully factorial design. Post-copulatory reproductive performance (egg number, hatching success and egg-to-adult viability) and testis size were measured in samples of males from each treatment combination at 2-week intervals (2, 4 and 6 weeks of age). We predicted that protein restriction in the adult diet would reduce male post-copulatory performance in early life, but may also result in a slower decline in reproductive ageing. This is compared to fully fed males that may have higher early reproductive performance but may suffer a steeper decline in fecundity due to faster ageing rates. However, we also expected that the effect of dietary protein would interact with larval nutrient availability and adult mating history. Larval nutrient availability has been shown to influence male condition and other traits in this species (e.g. Bonduriansky, 2007; Fricke *et al.*, 2015; Runagall-McNaull *et al.*, 2015), and frequent mating can increase ageing (e.g. Partridge & Farquhar, 1981; Perry & Tse, 2013) or decrease future fecundity in many species (e.g. Linklater *et al.*, 2007; Reinhardt *et al.*, 2011). We therefore predicted that larval nutrient limitation and adult mating may accentuate the effects of adult protein restriction.

Materials and methods

Study species

Telostylinus angusticollis is found on the east coast of Australia where they mate and feed on rotting bark of

native *Acacia longifolia* trees and the 'Brazilian coral tree hybrid', *Erythrina* × *sykesii*. Females generally aggregate around the most nutrient-rich areas, and these are guarded by the most competitive males. Hence, access to nutrients and mates varies widely between males (Bonduriansky, 2006). Wild-collected flies from Fred Hollows Reserve, Coogee, Sydney (33.91°S, 151.25°E), were mixed into existing laboratory stocks (originally from Fred Hollows Reserve, Coogee, Sydney) and then cultured for two generations prior to this experiment.

Experimental design

Six hundred eggs were collected from the stock flies and randomly assigned to nutrient-rich or nutrient-poor larval diet treatments (300 eggs per larval diet). The rich diet consisted of 30 mL sugar cane molasses (Conga Foods Pty. Ltd, Preston, Vic., Australia), 30 mL barley malt (Colonial Farms brand, Select Foods Pty. Ltd., Smithfield, NSW, Australia) and 32 g soy protein powder (Nature's Way brand; Pharm-a-care Pty. Ltd., Warriewood, NSW, Australia) per 1 L of coco peat, and the poor larval diet contained one-third of the nutrients per 1 L of coco peat (as described in Bonduriansky, 2007). All larvae were provided with 150 g of larval food per 50 eggs (six larval containers of 50 eggs per treatment), which was considered enough to limit any effects of density (E. L. Macartney, unpublished). Each larval container was kept at a constant temperature of 25°C with a light–dark cycle of 12 h and watered periodically.

After adult emergence, male flies within each of the larval diet treatments were randomly transferred into a mating manipulation and an adult diet treatment in a fully crossed factorial design, as shown in Fig. 1. Males from each larval container were evenly and randomly distributed among the adult treatments. For each replicate, one male and one female were housed together in a 440-mL container lined with damp coco peat (watered periodically to provide hydration) and covered with a stocking to provide ventilation. To manipulate mating history, half of the replicate males were prevented from mating by a mesh barrier until paired with a standardized virgin female for the fecundity assay (restricted mating), whereas the other males were allowed to mate by providing a 2 cm × 2 cm opening in the centre of the mesh to allow the male and female to pass freely from either side (*ad libitum* mating treatment). Individuals in the *ad libitum* mating treatment were provided with oviposition medium (rich larval diet that had been allowed to grow mouldy and then mixed) so the female could lay eggs, making her more receptive to mating.

To manipulate adult diet, half of the replicates were provided with a diet of brown sugar and yeast at approximately 3 : 1 ratio (fully fed diet), whereas the other half were given brown sugar alone (protein-restricted diet). A small amount of water was added to the fully fed diet in order to form a paste between the two nutrients and prevent the males from selectively choosing the concentrations of protein and carbohydrates consumed (as described in Adler *et al.*, 2013). The containers were kept at a constant temperature of 25°C with a 12-h light–dark cycle. Each combination of larval diet, adult diet and adult mating opportunity treatment consisted of 12 replicates (n_{total} = 96).

Four males from each treatment combination were randomly sampled at 2-week intervals for the fecundity assay (Fig. 1) and subsequent measurement of testis size. Males can live for over 8 weeks in the laboratory, and whereas males can become sexually mature within a couple of days post-eclosion, females can take up to 2 weeks to mature, making mating unlikely before 2 weeks of age (Adler *et al.*, 2013). Hence, samples were taken at 2, 4 and 6 weeks of age to represent relatively young, middle-aged and relatively old flies. Standardized virgin females (all reared on the rich larval diet) were produced in addition to treatment individuals for use in the assays. Eggs for the females were transferred at 2-week intervals to ensure that females were always between 2 and 3 weeks of age when mated to the treatment males.

Male fecundity assay

At ages 2, 4 and 6 weeks post-adult emergence, a subsample of treatment males (n = 4 from each treatment combination where possible) were transferred individually to a scintillation vial with a standardized virgin female for 6 h. Based on previous observations, 6 h was considered long enough for mating to occur as virtually all pairs mate at least once during a 6-h pairing, but mating behaviour and total number of matings for each pair were not recorded. After the 6-h cohabitation period, females were transferred to a 250-mL container with oviposition medium (premoulded rich larval food) to allow egg laying.

Egg output was counted after 72 h, and a random sample of 20 eggs where possible (egg number range: 9–20, mean = 19.31) were collected to test for egg hatching success and egg-to-adult viability. If no eggs had been laid, egg output was recorded as 0 and females were housed with oviposition medium for a further 42 h (allowing for measurement of egg hatching success and egg-to-adult viability in these replicates). The eggs were transferred onto damp filter paper (to allow observation of egg hatching) and placed on top of 100 g of the poor larval diet [as paternal effects are most pronounced when offspring are reared on a nutrient-poor larval diet (Bonduriansky & Head, 2007)].

After 42 h, the number of eggs to have hatched was recorded. Hatched eggs could be identified as empty egg shells under a Leica M60 stereomicroscope (Leica

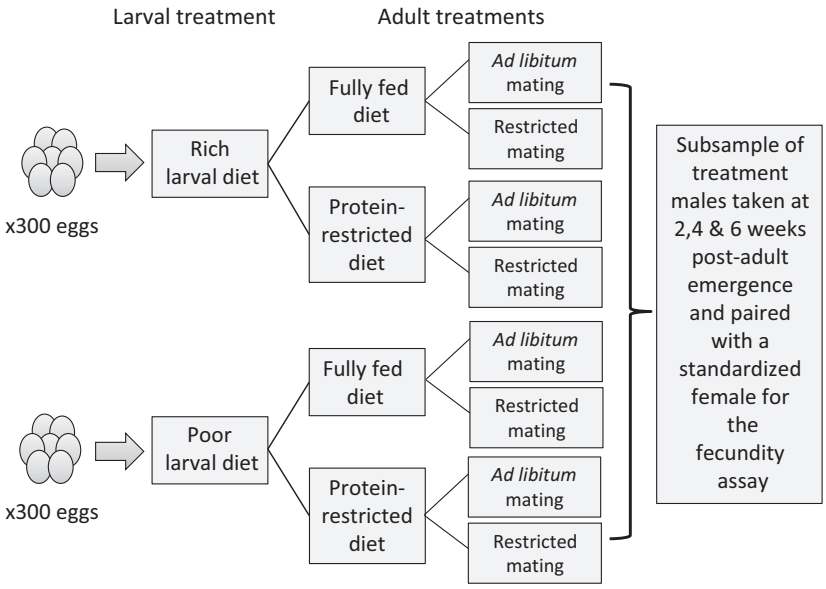

Fig. 1 Experimental design used to test for the effects of protein restriction in the adult diet, larval diet, mating history and age on male fecundity. Three hundred eggs per larval diet medium were transferred. Post-adult emergence, males were randomly allocated to an adult diet and mating treatment (n = 12 for each larval × adult treatment combination). Subsamples of these 12 males were taken at 2-week intervals for the reproductive performance assay (see text).

Microsystems, Heerbrugg, Switzerland). Larvae were then left to develop at a constant temperature of 25°C with a light–dark cycle of 12 h and watered periodically.

Egg-to-adult viability was determined by the number of adult flies to emerge from replicate containers (accounting for the number of eggs that were transferred).

Male testis size

A photograph of each male's thorax was taken at × 6.5 magnification. The testes were then removed under a Leica M60 stereomicroscope and photographed at × 25 magnification in a drop of Ph buffering solution on a microscope slide (enough to prevent desiccation but not enough to allow the testis to float). Photographs were taken using a Leica MZ16A stereoscope and a Leica DFC420-mounted camera. Measurements of testis area and thorax length were taken from images using Image J, version 1.47v (Rasband, 2015).

Statistical analysis

All models were initially fitted with adult diet, larval diet, mating history, age and all interactions as fixed effects. We tested the contribution of all interactions involving a particular categorical predictor to model fit by excluding this set of interactions and comparing the reduced model to the full model using a likelihood ratio test (see Chenoweth & Blows, 2005). These tests compare the variance explained by the reduced model to the variance explained by the full model based on a chi-squared statistic. If the contribution of a set of interactions to model fit was far from statistical significance based on a conservative cut-off ($P > 0.15$), these interactions were removed from the model. Otherwise, we interpreted the effects of particular significant interactions.

Treatment effects on focal male body size were analysed using linear regression. Some focal males died during the experiment (between 1 and 3 males per treatment combinations), reducing the sample sizes at 4 and 6 weeks of age (Fig. 2). Treatment effects on the probability of survival were analysed using a generalized linear model with binomial survival data (alive versus dead) and a logit-link function. Egg output was analysed using a generalized linear model with a 'quasi-Poisson' correction for overdispersion and a log-link function. Egg hatching success and egg-to-adult viability were analysed as binomial data using a generalized linear model and a logit-link function. Age^2 was included *post hoc* as a single fixed effect in the egg output model to test for a curvilinear effect but was not included in the final egg hatching success and egg-to-adult viability models as the quadratic term did not improve the models based on likelihood ratio tests. Testes size was analysed using a general linear model with thorax length included as a covariate. Both testis size and thorax length were standardized within larval diet to eliminate redundancy between the thorax

length covariate and the larval diet categorical predictor in this model. Male thorax and testis size (both standardized within larval diet) were initially included as covariates in egg output, egg hatching success and egg-to-adult viability models, but these covariates did not significantly improve any of the models and were therefore removed. *Post hoc* analysis of age on egg-to-adult viability was conducted using logistic regression and generalized linear models to test for differences in egg-to-adult viability with age within each adult diet, and in egg-to-adult viability between the adult diet treatments at each age point. All analyses were conducted using RStudio version 0.99.903 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Focal male body size and probability of survival

None of the interactions were strongly supported so were removed from the models. The rich larval diet produced significantly larger focal males (mean = 4.8 mm, SE = 0.03) compared to the poor larval diet (mean = 2.57 mm, SE = 0.02) (Table 1). Males assigned to different adult diet and mating treatments did not differ significantly in body size (Table 1). Males from the fully fed adult diet suffered a near-significantly higher probability of death before reaching 6 weeks of age (Table 1, Fig. 2). Neither larval diet nor mating treatment affected male survival probability (Table 1).

Egg output

Egg output ranged from 0 to 125 eggs laid in the first 72 h after mating (mean = 36.85, SE = 3.81). None of the interactions were strongly supported so were removed from the model. Male age and age^2 significantly influenced female egg output, with egg output peaking when males were 4 weeks of age (Table 2, Fig. 3).

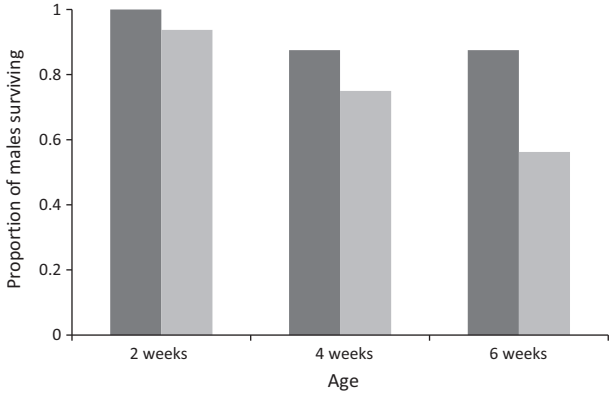

Fig. 2 Bar graph of the proportion of males surviving at each age group. The dark grey bars represent males from the protein-restricted adult diet, and the light grey bars represent males from the fully fed adult diet.

Egg hatching success

All interactions were strongly supported and therefore remained in the model (Table 2). We detected a significant adult diet × larval diet × mating treatment × age interaction (Fig. 4). There is a large variance in egg hatching success depending on the environmental combination as well as some large standard errors, making this four-way interaction difficult to interpret. However, it is clear that egg hatching success changes with male age and the age-dependent pattern is context dependent. We also detected a larval diet × adult diet × mating interaction, an adult diet × age interaction and a main effect of adult diet. However, lower-level interactions and main effects must be interpreted with caution given the significant four-way interaction.

Egg-to-adult viability

All interactions with all the main effects were strongly supported and therefore remained in the model (Table 2). We detected a significant three-way larval diet × mating treatment × age interaction (Fig. 5). When males could mate throughout their life, males from both the poor larval diet and the rich larval diet had increased egg-to-adult viability from 2 weeks of age to 4 weeks of age. However, males from the poor larval diet suffered a decline in egg-to-adult viability at 6 weeks of age compared to males from the rich larval diet, although egg-to-adult viability was higher at 6 weeks of age in the single male from the rich larval diet that survived to that age. Restricted mating males from the poor larval diet had lower egg-to-adult viability at both two and 4 weeks of age. However, they had a sharp increase in egg-to-adult viability at 6 weeks of age where they achieved the same level of egg-to-adult viability as males from the rich larval diet.

Table 1 The effects of male age, mating history, larval and adult diet on focal male body size and survival.

	Thorax length*		Survival probability†	
	Estimate	*P*	Estimate	*P*
Age	0.027	0.083	**−0.594**	**0.004**
Adult diet	0.000	0.996	**−1.21**	**0.052**
Larval diet	**3.023**	**< 0.001**	0.837	0.152
Mating	0.059	0.239	−0.520	0.382

*d.f. = 71.
†d.f. = 9.
Values in bold are statistically significant.

Table 2 The effects of male age, mating history, larval and adult diet on egg output, egg hatching, egg-to-adult viability and testis size.

	Egg output*		Egg hatching†		Egg-to-adult viability‡		Testis size§	
	Estimate	*P*	Estimate	*P*	Estimate	*P*	*F*	*P*
Thorax size	–	–	–	–	–	–	0.295	0.589
Adult diet	−0.260	0.192	**2.366**	**0.014**	**1.503**	**0.035**	0.760	0.386
Larval diet	0.072	0.709	0.262	0.723	−0.762	0.237	0.036	0.85
Age	**1.630**	**< 0.001**	0.063	0.61	**0.338**	**0.004**	1.683	0.199
Age^2	**−0.180**	**< 0.001**	–	–	–	–	–	–
Mating	0.063	0.744	0.164	0.841	−1.616	0.085	1.923	0.164
Adult diet × larval diet	–	–	−2.121	0.083	0.742	0.427	–	–
Adult diet × age	–	–	**−0.529**	**0.011**	**−0.434**	**0.007**	–	–
Adult diet × mating	–	–	−0.905	0.51	0.228	0.845	–	–
Larval diet × age	–	–	0.285	0.133	0.211	0.158	–	–
Larval diet × mating	–	–	0.133	0.902	**3.250**	**0.003**	–	–
Age × mating	–	–	−0.092	0.646	0.188	0.381	–	–
Adult diet × larval diet × mating	–	–	**8.613**	**0.002**	−0.311	0.836	–	–
Adult diet × age × mating	–	–	0.469	0.147	0.191	0.483	–	–
Adult diet × age × larval diet	–	–	0.191	0.514	−0.209	0.35	–	–
Larval diet × age × mating	–	–	−0.082	0.766	**−0.599**	**0.015**	–	–
Adult diet × larval diet × age × mating	–	–	**−1.866**	**0.001**	0.023	0.949	–	–

*d.f. = 76.
†d.f. = 63.
‡d.f. = 63.
§d.f. = 65.
Values in bold are statistically significant.

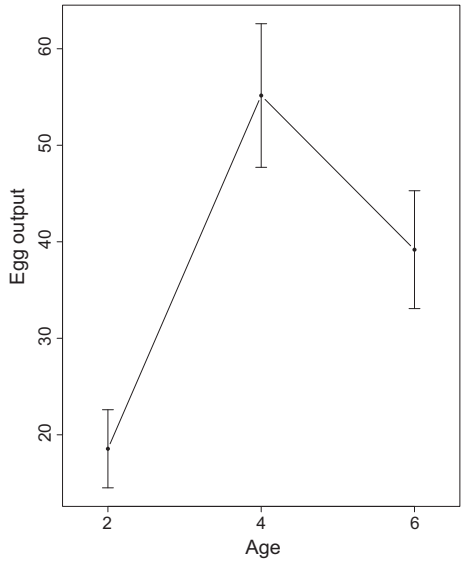

Fig. 3 Line plot showing the effect of male age on the mean number of eggs laid by the standardized females (± SE).

We also detected a two-way interaction of adult diet and age (Fig. 6). Males from the protein-restricted diet had significantly lower egg-to-adult viability at 2 weeks of age compared to fully fed males ($Z_{20,21} = 3.492$, $P < 0.001$); there was no difference between egg-to-adult viability at 4 weeks of age ($Z_{24,23} = -0.210$, $P = 0.834$), but egg-to-adult viability from protein-restricted males significantly exceeded that of fully fed males at 6 weeks of age ($Z_{16,15} = -3.410$, $P < 0.001$). Linear regression analysis within adult diet showed that it is the protein-restricted diet driving the interaction, with a significant increase in egg-to-adult viability with male age ($t_{35} = 3.221$, $P = 0.003$). There was no significant change in egg-to-adult viability with age in fully fed males ($t_{25} = -0.086$, $P = 0.932$).

There was also a significant two-way interaction of larval diet × mating treatment as well as significant main effects of age and adult diet. However, these effects need to be interpreted with caution due to their higher-level interactions.

Testes size

None of the interactions were strongly supported and so were removed from the model (Table 2). None of the main effects or thorax size (standardized within larval diet) had a significant effect on testis size.

Discussion

We investigated how adult protein restriction affects male post-copulatory performance, and asked how the effects of protein restriction are modulated by male age, developmental nutrition and mating history. We show

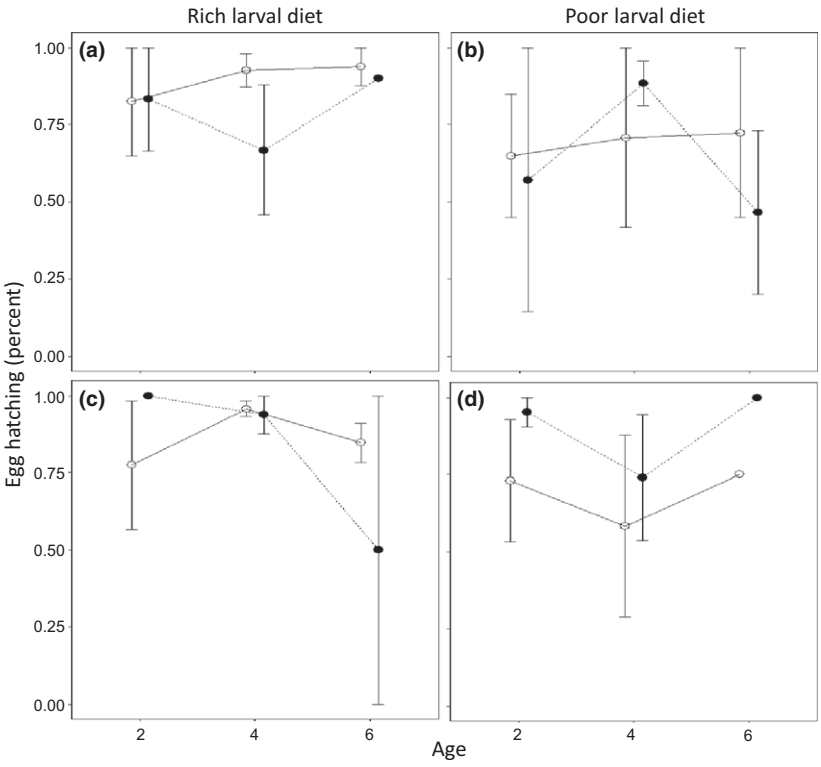

Fig. 4 Interaction of male age and adult diet on mean egg hatching success (± SE) with males grouped by the *ad libitum* mating treatment and rich larval diet (a), *ad libitum* mating treatment and poor larval diet (b), restricted mating treatment and rich larval diet (c) and restricted mating treatment and poor larval diet (d). The closed circles with dotted lines represent males from the fully fed adult diet, and the open circles with solid lines represent males from the protein-restricted adult diet.

that protein restriction does not result in an overall decline in reproductive performance, but that protein can have important age-dependent effects on male fecundity and offspring viability. We found that male fecundity (the number of eggs laid by females) peaked at 4 weeks of age, but was not affected by protein content of the male adult diet, nor by larval diet or mating history. Likewise, there was no evidence that protein-restricted males had consistently lower egg hatching success. However, we observed age-dependent effects of adult dietary protein on egg-to-adult viability of offspring: offspring viability remained constant with age in fully fed males but increased more than two-fold from 2 to 6 weeks of age in protein-restricted males. Thus, protein-restricted males exhibited reduced post-copulatory performance in early life but also improved with age, compensating for their poor performance in early life.

Protein restriction slows actuarial ageing and extends lifespan in both males and females while also

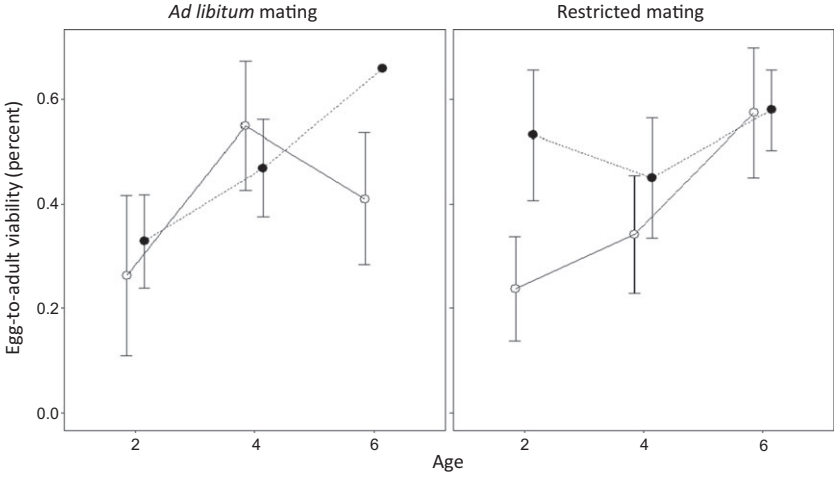

Fig. 5 Interaction of larval diet, age and mating on mean egg-to-adult viability (± SE). The closed circles with dotted lines represent males from the rich larval diet, and the open circles with solid lines represent males from the poor larval diet.

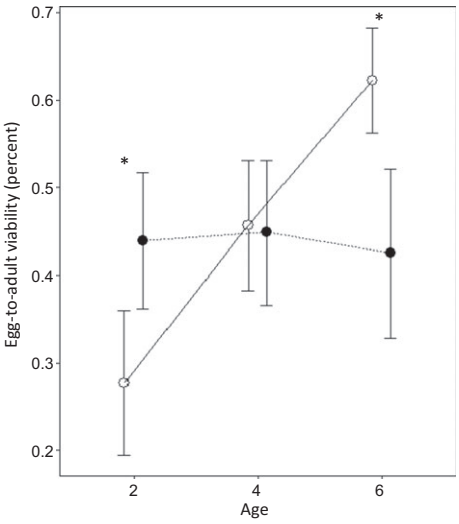

Fig. 6 Interaction of adult diet and age on mean egg-to-adult viability (± SE). The closed circles with dotted lines represent males from the fully fed adult diet, and the open circles with solid lines represent males on the protein-restricted adult diet.
* indicates $P < 0.001$.

compromising female reproductive performance (reviewed in Merry, 1995; Masoro, 2005; Partridge *et al.*, 2005), but effects of protein restriction on male reproduction are poorly understood. We observed complex, age- and context-dependent effects of protein restriction on post-copulatory reproductive performance in males. The interaction of larval diet, mating history and age with adult dietary protein resulted in complex patterns in egg hatching success, suggesting that protein restriction in the adult diet can either enhance egg hatching success (Fig. 4a) or reduce egg hatching success (Fig. 4d) depending on male larval diet and mating history. Mating history, larval diet and age also interacted to influence offspring egg-to-adult viability, with males reared on a nutrient-poor larval diet producing less viable offspring either early in life (Fig. 5 left panel) or later in life (Fig. 5 right panel) depending on mating treatment. Although the causes of the patterns are not known, these results show that male post-copulatory performance is highly context dependent and that dietary protein can interact with other factors in the male environment to influence aspects of male reproductive success.

Nonetheless, adult dietary protein had clear age-dependent effects on a key aspect of male post-copulatory performance: fully fed males produced more viable offspring than protein-restricted males early in life, whereas, later in life, protein-restricted males produced more viable offspring than fully fed males. This finding is consistent with the expectation that dietary protein enhances performance in early life at the expense of performance at later ages. However, neither fully fed nor protein-restricted males appeared to suffer reproductive deterioration with age (i.e. reproductive ageing) in their ability to produce viable offspring, although our results suggest that fully fed males are more likely to die earlier, reflected in a higher proportion of males dying before reaching 6 weeks of age. This is consistent with previous studies where fully fed individuals suffer faster actuarial ageing rates (Merry, 1995; Magwere *et al.*, 2004; Masoro, 2005). Despite this effect of adult diet on mortality rate, we found no evidence of selection on male quality, in that surviving fully fed males did not exhibit enhanced performance at 6 weeks of age relative to protein-restricted males. However, the males that died younger may have suffered rapid reproductive ageing that may have been detected if measured under a finer time scale. Nonetheless, our results suggest differing reproductive strategies depending on adult dietary protein. Neither fully fed nor protein-restricted older males appeared to allocate resources away from the components of the ejaculate that influence offspring viability, and protein-restricted males may have invested more in offspring viability as they aged. Males at four and 6 weeks of age induced greater egg output compared to males at 2 weeks of age, and thus, the enhanced egg-to-adult viability at 6 weeks of age in protein-restricted males may result in a disproportionate increase in offspring produced. However, it should be noted that we did not examine precopulatory reproductive success (i.e. performance in competition for territories and mates, or if these males achieved greater precopulatory success with the standardized virgin females during the fecundity assay). We therefore cannot assess effects of protein restriction on overall male reproductive performance, or the potential for differences in mating behaviour with the standardized female to drive the effects on offspring viability.

It is possible that males in the protein-restricted treatment consumed more food in order to compensate for the lack of protein, thereby increasing their total energy intake (Sørensen *et al.*, 2008), or they may have exhibited a form of terminal investment (Davison *et al.*, 2014). It has been hypothesized that when individuals are limited in their ability to reproduce due to limited energetic resources, reproductive effort will increase with age. This is due to later maturation and longer lifespans in resource-limited individuals compared to the earlier maturation and shorter lifespans of individuals with high resource availability (Gadgil & Bossert, 1970). Individuals with a shorter lifespan also tend to reproduce at an earlier age (Rose, 1984; Roitberg *et al.*, 1993; Davison *et al.*, 2014). Thus, our findings that fully fed males achieved high post-copulatory performance early in life and were more likely to die at a younger age, and that protein-restricted males had increasing reproductive performance in their ability to produce viable offspring and were more likely to survive until 6 weeks of age, are consistent with these reproductive

investment hypotheses. Although the cause of the observed difference in reproductive performance with age between fully fed and protein-restricted males is not known, our results suggest that these males are pursuing different reproductive strategies.

Overall, our results suggest a far more complex story than a simple reallocation of nutrients away from reproduction in protein-restricted males. The context dependence and age dependence of protein restriction, with no clear overall reduction in any of the post-copulatory traits measured, challenge the hypothesis that resources are reallocated away from reproduction in order to extend lifespan (Holliday, 1989; Shanley & Kirkwood, 2000; Davison *et al.*, 2014). Both Zajitschek *et al.* (2012) and Adler *et al.* (2013) found that the effects of adult protein consumption on male lifespan and reproduction are context dependent, varying with male mating status in *Teleogryllus commodus* (Zajitschek *et al.*, 2012) and with larval diet and social environment in *T. angusticollis* (Adler *et al.*, 2013). Also, Fricke *et al.* (2008) demonstrated that the effects of adult protein availability on male *Drosophila melanogaster* reproductive performance are not linear, with male reproductive performance peaking at intermediate protein levels. Taken together, our results and the studies above suggest that adult protein consumption is important for male reproductive performance and that this extends to paternal investment into components of the ejaculate that influence offspring viability. However, the effects of dietary protein on male reproduction appear to be nonlinear, strongly dependent on male age, and perhaps also influenced by other factors such as developmental diet and social environment.

These complex results may be due to differing nutritional requirements between male and female reproductive traits, and the differing costs of investment in these traits. For example, the primary cost of reproductive investment for females is the production of ovules, and protein is required for oogenesis (Chippindale *et al.*, 1993; Adler *et al.*, 2013). Therefore, a reduction in dietary protein is likely to significantly reduce synthesis of eggs. In contrast, males can invest in multiple pre- and post-copulatory traits that have differing protein requirements and differing costs of investment (Fricke *et al.*, 2008; Maklakov *et al.*, 2008; Gosden & Chenoweth, 2011; Zajitschek *et al.*, 2012; Moatt *et al.*, 2016). For example, there are many proteins in the semen that have a range of functions in fertilization and sperm competition (Chapman, 2001; Perry *et al.*, 2013), and these are likely to require protein to manufacture. On the other hand, other mechanisms may be involved in influencing offspring viability such as semen- and sperm-borne RNA, and these may require little protein to synthesize but may be influenced by other environmental factors (Rassoulzadegan *et al.*, 2006; Eaton *et al.*, 2015; Skinner, 2015). Therefore, males may not reallocate energetic resources away from reproduction as a whole when adult dietary protein is limited, but may alter their reproductive strategies, including the scheduling of reproductive investment with age.

Our findings show that dietary protein plays an important role in the scheduling of male post-copulatory investment. Protein can have complex interactions with other ecologically relevant factors to influence age-dependent changes in post-copulatory reproductive performance, including transgenerational effects on offspring viability. Thus, in order to understand how protein restriction influences male reproductive performance, it will be necessary to investigate effects on multiple pre- and post-copulatory reproductive traits at multiple ages and under a range of ecologically relevant contexts.

Acknowledgments

We thank Amy K. Hooper and Nathan W. Burke for discussing ideas and providing advice. This work was funded by an Australian Research Council Future Fellowship (FT120100274). We have no conflicts of interest to declare.

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Data deposited at Dryad: https://doi.org/10.5061/dryad.g056v

Received 18 December 2016; revised 27 March 2017; accepted 3 April 2017