

# Male reproductive performance: Influence of age, sex and diet on fecundity and offspring quality

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# Male reproductive performance: Influence of age, sex and diet on fecundity and offspring quality

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## PLEASE TYPE

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Male reproduction bears an energetic cost, making reproductive performance dependent on resource availability and the previous utilisation of those resources. This thesis examines how male post-copulatory reproductive performance responds to different condition-determining factors through two experimental studies, using the neriid fly *Telostylinus angusticollis*. Offspring quantity, trans-generational paternal effects and male reproductive morphology were used as measures of reproductive performance, making this one of the few studies to include trans-generational effects when examining male reproductive plasticity. Chapter one aimed to determine any interactions of determinants of male condition at the larval and adult stage, as well as male mating history on reproductive performance. It is demonstrated that protein consumed by males at the adult stage interacts with male age to influence larval viability of the offspring. Moreover, it was found that female fecundity stimulation induced by the male shows complex condition-dependent responses to male environment and age. Chapter two aimed to determine if there was a cost to frequent mating on male reproductive performance. It was found that males that had mated frequently could achieve similar reproductive performance as that of virgin males. It was also found that larval viability is influenced by male accessory gland size and that male mating history influences accessory gland size, although no effect of mating history on larval viability was found. This thesis provides insight into how male reproductive performance responds to varying environments and determinants of male condition. It also demonstrates that male reproductive performance should not be limited to offspring quantity, but should include trans-generational paternal effects such as offspring viability and condition.

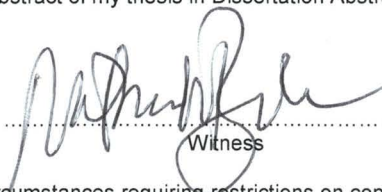
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## Abstract

Male reproduction bears an energetic cost, making reproductive performance dependent on resource availability and the previous utilisation of those resources. This thesis examines how male post-copulatory reproductive performance responds to different condition-determining factors through two experimental studies, using the neriid fly *Telostylinus angusticollis*. Offspring quantity, trans-generational paternal effects and male reproductive morphology were used as measures of reproductive performance, making this one of the few studies to include trans-generational effects when examining male reproductive plasticity. Chapter one aimed to determine any interactions of determinants of male condition at the larval and adult stage, as well as male mating history on reproductive performance. It is demonstrated that protein consumed by males at the adult stage interacts with male age to influence larval viability of the offspring. Moreover, it was found that female fecundity stimulation induced by the male shows complex condition-dependent responses to male environment and age. Chapter two aimed to determine if there was a cost to frequent mating on male reproductive performance. It was found that males that had mated frequently could achieve similar reproductive performance as that of virgin males. It was also found that larval viability is influenced by male accessory gland size and that male mating history influences accessory gland size, although no effect of mating history on larval viability was found. This thesis provides insight into how male reproductive performance responds to varying environments and determinants of male condition. It also demonstrates that male reproductive performance should

not be limited to offspring quantity, but should include trans-generational paternal effects such as offspring viability and condition.

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## **General Introduction**

This thesis focuses on the plasticity of multiple facets of male post-copulatory reproductive performance due to environment and male condition, including effects on offspring quantity and trans-generational paternal effects. My aim was to gain a thorough understanding of how male environment and male condition drives diversity in reproduction between individuals and across that individual's lifetime, as well as diversity in offspring sired.

All individuals have a limited resource pool to invest into reproduction and the size of this resource pool at a given time is determined by nutrients acquired from the diet and the amount of resources the individual has previously invested (Boggs 1981, Stearns 1989). This can result in the plasticity of various life-history traits including reproductive performance. A lot of research has focussed on female reproductive performance and plasticity due to the more obvious costs of investment. However, I am particularly interested in male reproductive investment and plasticity.

Traditionally, male reproduction was thought to be cheap and it was believed that reproductive output should increase with increased insemination of females (Bateman 1948). However, evidence suggests that investment into ejaculate components used for post-copulatory success (particularly for males faced with sperm competition) bears an energetic cost (Dewsbury 1982, Simmons 2005). This makes it likely that investment into reproduction may be subject to condition-

dependent responses (Dewsbury 1982, Wedell et al. 2002, Parker and Pizzari 2010).

Previously, males were assumed to transfer only sperm, so male reproductive performance depended on genetic quality and perhaps sperm number and performance. Recently, people realized that (1) there is more to sperm than genes, because the sperm genome can be epigenetically modified and these epigenetic factors can influence offspring (Bonduriansky and Day 2009, Tal et al. 2010, Crean and Bonduriansky 2014), and (2) that the seminal fluid contains molecules that can influence female fecundity and offspring phenotype and fitness (Chapman 2001, Wolfner 2002, Poiani 2006, Simmons 2011). Therefore, male reproductive performance can include components that can influence fecundity stimulation and paternal effects. The size of the males' testes (used as a proxy for sperm production) and the size of the accessory glands (used as a proxy for the amount of seminal fluid proteins) can also be measured to determine changes in male reproductive potential. In this thesis, I therefore include multiple measures of male reproductive performance, including fecundity stimulation, egg hatching success and trans-generational paternal effects on offspring viability and phenotype, as well as measures of male testis and accessory gland size\*. This provides a more thorough interpretation of the effects of condition-determining factors on male reproductive performance.

\*Due to a freezer malfunction during experiment one, male accessory gland size was unable to be measured for this experiment.



Males of *Telostylinus angusticollis* are an excellent study species to examine reproductive plasticity as multiple traits have been demonstrated to be highly condition dependent. Male body size is influenced by the amount of nutrients found in the larval diet (Bonduriansky 2007, Sentinella et al. 2013), and adult diet and social environment have also been shown to influence aspects of male reproduction including male-induced egg-output and egg-to-adult viability (Adler and Bonduriansky 2013, Adler et al. 2013). The condition of the male due to nutrients in the larval diet has also been shown to be conferred to his offspring through a non-genetic paternal effect: large males reared on a nutrient rich larval diet produce large offspring, and small males reared on a nutrient poor larval diet produce low condition offspring (Bonduriansky and Head 2007). Hence, *T. angusticollis* males have been shown to display a high degree of plasticity in aspects of reproduction – making them an excellent study system to continue to investigate condition-determining effects on male reproductive performance.

It is important to determine the interactions of multiple variables on male reproductive performance as this provides us with more ecologically relevant information as to how males respond to multiple condition-determining factors, rather than the standard one or two factor manipulations. It is known that nutrients acquired at different life stages can influence aspects of male reproductive performance (Boggs 1981, Droney 1998, Fricke et al. 2008, Carsten-Conner et al. 2010, Adler et al. 2013), and that previous investment into reproduction can alter future reproductive performance (Hihara 1981, Linklater et al. 2007, Reinhardt et al. 2011). Male reproductive performance can also change

with male age due to inevitable changes in male condition as well as due to a potential trade-off between reproductive investment and somatic maintenance (Merry 1995, Partridge and Brand 2005). However, the interactions of all these factors have not been examined and it is likely that the interactions of multiple condition-determining factors will provide a different insight into the effects on male reproductive performance compared to examining each factor individually. Therefore, chapter one examined how male reproductive performance is influenced by a range of condition-determining factors (nutrients in the larval and adult diet and age) as well as male mating history. My objective was to understand the relative importance and interactive effects of male diet and mating history on male reproductive performance, and whether these effects change with male age. This was completed using a fully-crossed factorial design and mating a random sample of males of different ages to standard virgin females in order get measures of reproductive performance.

A high degree of plasticity of reproductive performance was found in chapter one and this led me to consider if the effects found in chapter one and the paternal effects on offspring condition found by Bonduriansky and Head (2007) could be depleted after frequent mating. There is evidence to suggest that the responses found in chapter one are quite often driven by components in the semen (Chapman 2001, South and Lewis 2011), and research conducted by Crean et al. (2014) indicated that the paternal effects on offspring condition previously found in *T. angusticollis* are likely transferred via components in the semen rather than epigenetic changes to the DNA in the sperm. Hence, if reproductive performance is

highly condition dependent (as found in chapter 1) and bears an energetic cost of investment, it could be predicted that the components (such as seminal fluid proteins) driving the responses found could be subject to depletion after frequent mating. A depletion of ejaculate and a corresponding decrease in reproductive output has been found in other species (Halliday and Houston 1978, Rutowski 1979, Hihara 1981, Linklater et al. 2007, Sirot et al. 2009); however this needs to be tested in other study systems with the inclusion of multiple measures of reproductive performance including trans-generational paternal effects. Therefore, chapter two tested for a cost of mating on male reproductive performance by mating some males frequently over a fixed period of time and keeping the other males virgins, then mating them to standard virgin females to attain measures of male reproductive performance.

Overall, this thesis presents two chapters aimed to disentangle the complex effects of male environment and condition on the plasticity of post-copulatory reproductive performance, including fecundity stimulation and trans-generational effects on offspring quality. It is important to determine interactions of different factors that may influence male reproductive performance in order to further understand sources of variation in natural populations. It is also important to test ideas that have become prevalent in the current literature, such as the cost of mating to males on future reproductive performance. The inclusion of trans-generational paternal effects as part of male reproductive performance is necessary in gaining a thorough understanding of how extensive the effects of male condition-determining factors are on male reproduction, and the trans-

generational effects on offspring further contribute as a source of variation in natural populations.

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# CHAPTER ONE

## **Age dependent effects of male nutritional and mating environment on female fecundity stimulation and offspring viability**

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ELM, AC and RB conceived and designed the experiment, ELM carried out the experiment, analysed the data and wrote the manuscript, AC and RB provided supervision and guidance throughout the experiment, analysis and writing process.

## Abstract

Paternal effects are increasingly recognized as widespread and important, but we know little about how paternal effects relate to other components of male reproductive strategy, or how multiple environment and condition determining factors interact to influence paternal effects and male reproductive performance. Fecundity stimulation and offspring viability are two key components of male post-copulatory performance that are likely to be influenced by the availability of dietary resources such as protein, as well as age-related changes in condition. We manipulated male larval and adult diet quality as well as mating opportunity in the neriid fly *Telostylinus angusticollis*, and examined age-specific effects of these variables on the males' ability to stimulate oviposition in their mates, hatching success of the eggs, and juvenile viability of offspring. It was found that the interaction of male age, diet and past mating opportunity affected female oviposition rate. There were no male effects on egg hatching success detected. However, we found that increased consumption of protein by adult males enhanced offspring juvenile viability at young ages but reduced offspring juvenile viability at older ages. Environment and age also influenced testis size, but variation in testis size did not drive any of the other responses observed, suggesting a possible role for accessory-gland proteins. Our findings show that dietary protein regulates age-related changes in a males capacity to stimulate oviposition, and in paternal effects on offspring viability.

## Introduction

Trivers (1972) defined parental investment as “any investment by the parent in an individual offspring that increases the offspring's chance of surviving at the cost of the parent's ability to invest in other offspring.” Both maternal and paternal care are prime examples of this. It is widely accepted that the maternal environment can have a significant effect on offspring traits and female reproductive investment strategies (Mousseau and Dingle 1991, Rossiter 1996, Bonduriansky and Head 2007, Crean and Marshall 2009, Adler et al. 2013). However, it is now becoming apparent that the paternal environment can also influence offspring traits and male reproductive investment, even without any direct paternal provisioning through nuptial gifts or spermatophores (Islam et al. 1994, Bonduriansky and Head 2007, Adler and Bonduriansky 2013, Crean and Bonduriansky 2014). This can occur through epigenetic changes in the sperm (Anway et al. 2005, Rando 2012, Soubry et al. 2014), or through non-genetic components of the seminal fluid (Linklater et al. 2007, Wigby et al. 2009, Perry and Rowe 2010, Simmons 2011). Thornhill (1976) argued that some traits blur the distinction between paternal investment and traits that function in sperm competition. For example, seminal fluid proteins (SFPs) are known to play a role in sperm competition by altering female physiology so as to increase egg laying and latency to re-mate in *Drosophila melanogaster* (Hihara 1981, Chapman et al. 1995, Chapman 2001). However, SFPs can also increase offspring viability, and thereby mediate paternal effects that may represent a form of paternal investment (Wolfner 1997, Chapman 2001, Avila et al. 2011). Paternal influences on reproductive output and offspring viability may represent an investment strategy that imposes an energetic cost and may depend on the condition of the male, determined by environmental parameters and age.

In many different mating systems, a large body size in males is representative of high male condition and is particularly important for pre-copulatory selection. This can be influenced by nutrients in the diet (Yuval et al. 1998, Blanckenhorn 2000, Bonduriansky 2007). In holometabolous insects, body size is determined at the larval stage with some energetic reserves gained at this stage likely allocated to reproduction (Boggs 1981). Males of holometabolous insects reared on a nutrient rich larval diet are of larger body size compared to that of males reared on a nutrient poor larval diet (Boggs 1981, Bonduriansky and Head 2007), and this condition can be conferred to the males' offspring through a non-genetic mechanism (Bonduriansky and Head 2007, Valtonen et al. 2012). This non-genetic paternal effect has been shown in two Diptera species that do not provide any conventional form of paternal provisioning (such as spermatophores or nuptial gifts): *Telostylinus angusticollis* (Bonduriansky and Head 2007, Adler and Bonduriansky 2013) and *Drosophila melanogaster* (Valtonen et al. 2012). In these species, males that develop on a resource-rich larval diet produce larger offspring. This suggests that even without direct provisioning, males are still able to confer a fitness benefit to their offspring through non-genetic paternal effects, and in *T. angusticollis* this is possibly due to components found in the semen, such as seminal fluid proteins (Crean et al. 2014).

Most studies to date have only examined effects of one or two variables on male reproductive performance, but performance can depend on multiple condition-determining variables (Zajitschek et al. 2012, Adler and Bonduriansky 2013, Adler et al. 2013). Research has demonstrated the importance of nutrients in the adult diet on male reproductive performance (Droney 1998, Fricke et al. 2008, Carsten-



Conner et al. 2010) and in females, it is also commonly accepted that reproduction and lifespan can trade-off against each other due to protein consumption at the adult stage (Boggs and Ross 1993, Merry 1995, Shanley and Kirkwood 2000, Partridge and Brand 2005). If male reproduction is costly, it is likely that inevitable changes in male condition may also be enhanced by environment due to a potential trade-off with reproduction and somatic maintenance, and male reproductive performance may change as a function of male age. Both Adler et al. (2013) and Zajitschek et al. (2012) found that the effects of adult diet (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 *Telostylinus angusticollis* (Adler et al. 2013); therefore emphasising the need to consider as many ecologically relevant variables as possible when investigating parental effects and reproductive investment in an ecologically relevant context.

*Telostylinus angusticollis* (Diptera: Neriidae) is a polyandrous species with highly condition dependent body size and shape. The amount of dietary protein and carbohydrates available in the larval diet results in significant changes in male body size, with males fed on high protein and carbohydrate diets showing a significant increase in body size compared to males fed on low protein and carbohydrate diets (Bonduriansky 2007, Sentinella et al. 2013). The male condition resulting from nutrients in the larval diet has been shown to be conferred to the male's offspring, demonstrating a transgenerational effect of male environment (Bonduriansky and Head 2007). The combined effects of male diet at the larval and adult stage and social environment on male reproductive

performance have been explored in *Telostylinus angusticollis* (Adler and Bonduriansky 2013, Adler et al. 2013), however the interaction with male age has not been examined.

We investigated how a range of paternal environmental factors (larval and adult diet as well as mating history) interact together to affect male reproductive performance (egg output, egg hatching success and egg-to-adult viability). We also tested for interactions of these factors with male age. We expected that the combination of larval diet (rich and poor concentrations of protein and carbohydrates) and adult diet (protein rich or protein poor) as well as male mating history (non-mated or frequently mated) would interact to differentially affect egg-output, egg hatching success and egg-to-adult viability and that these responses would differ with male age. Due to the cost of investment in reproduction, we expected that males from nutrient poor larval and protein poor adult diets would have fewer resources to allocate and perhaps suffer reduced reproductive performance compared to males from nutrient rich larval diets and the protein rich adult diets. We also predicted that males from the mated treatments may suffer a steeper decline in reproductive performance with age as theory predicts that males that invest heavily in early life reproduction may suffer a cost to reproduction later in life (Cameron et al. 2007). Males from the nutrient poor diets may need longer to accumulate resources from the adult diet and may increase reproductive performance with age in a form of terminal investment (Davison et al. 2014). We also examined the effect of environment and age on testis size (used as a proxy for sperm production) and if testis size mediated any of the effects in reproductive investment. This study is one of the first to investigate how a range of

condition determining parameters interact together to affect both fecundity stimulation and paternal effects on offspring viability.

## **Materials and Methods**

### Study species

*Telostylinus angusticollis* is found on the east coast of Australia where they mate and feed on rotting bark. 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 among males (Bonduriansky 2006). Males transfer no spermatophore or nuptial gift; rather, sperm is packaged into bundles prior to copulation and the spermatids dissociate after insemination (EM, unpublished data).

Wild-collected flies from Fred Hollows Reserve, Coogee, Sydney (33.91° S, 151.25° E) supplemented lab stock flies (originally from Fred Hollows Reserve, Coogee, Sydney) and cultured for two generations prior to this experiment.

### Manipulation of male condition

To obtain males in high- and low-condition, 600 eggs were collected from the stock flies and randomly assigned to a rich larval diet or a poor larval diet (300 eggs per larval diet). Both diets consisted of coco-peat, water, molasses, liquid malt barley and protein powder, with the poor diet containing 1/3 of the nutrients per litre of coco-peat compared to the rich diet (as described in Bonduriansky (2007)). All larvae were given the same quantity of larval food but with altered nutrients

depending on the treatment. 150g of larval food per 50 eggs (6 larval containers of 50 eggs per treatment) was considered enough to limit any effects of density, based on previous studies on this species (EM, unpublished data). Each larval container was kept at a constant temperature of 25°C with a light-dark cycle of 12hrs and watered periodically.

After emergence, male flies within each of the larval diet treatments were randomly transferred into a mating opportunity manipulation and 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 440mL container lined with damp coco-peat (watered periodically to provide hydration) and covered with a stocking to provide ventilation. To manipulate mating opportunity, in half the replicates males were prevented from mating (non-mating treatment) by a mesh barrier, while in the other half of replicates a 2cm x 2cm square was cut in the centre of the mesh to allow the male and female to pass freely from either side and mate at will (mating treatment). Individuals in the mating treatment were provided with oviposition medium (rich larval diet that had been allowed to grow mould) so the female could lay eggs, making her more receptive to mating. Males will mate frequently with the same female even when no other females are present (Bath et al. 2012).

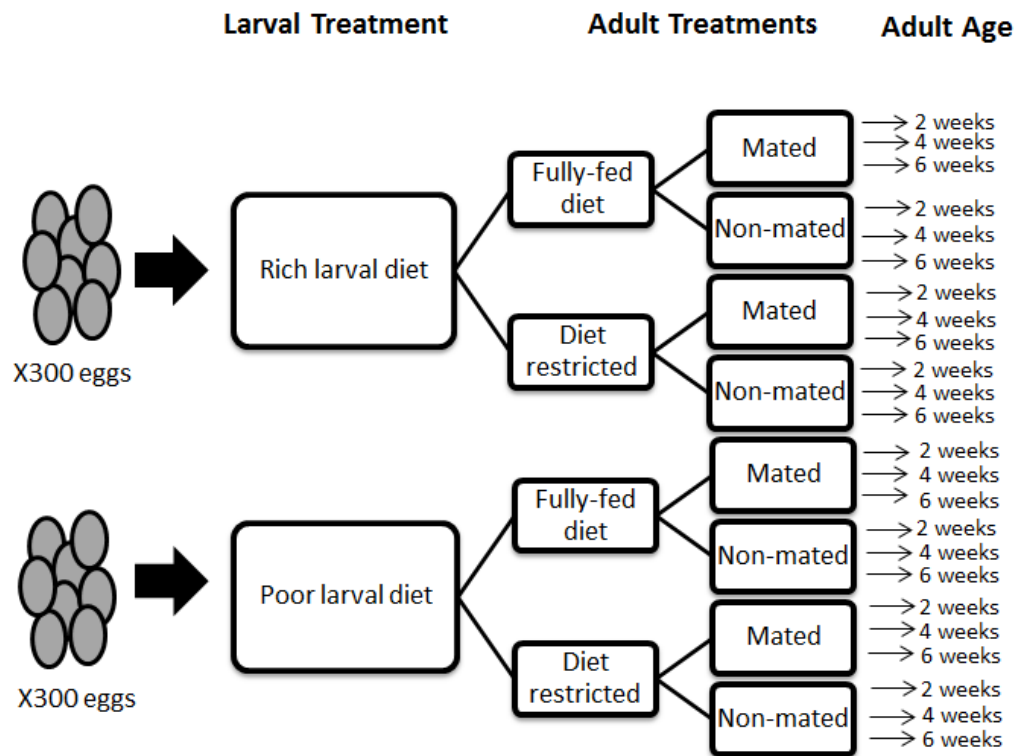
To manipulate adult diet, half the replicates were provided with a diet of brown sugar and yeast at approximately a 3:1 ratio (protein rich diet) while the other half were given brown sugar alone (protein poor diet). A small amount of water was added to the protein rich adult diet in order to form a 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)). Adult food was replaced when it moulded. The containers were kept at a constant temperature of 25°C with a 12 hour light-dark cycle. Any females that died during the experiment were replaced within 24 hours. Each combination of larval diet, adult diet and adult mating opportunity treatment consisted of 12 replicates ( $n_{\text{total}} = 96$ ).

Subsamples of four males from each treatment combination were taken at two-week intervals for the male reproductive performance assay (**Fig. 1**). Males are known to reach sexual maturity a few days after eclosion; therefore the samples were taken at 2, 4 and 6 weeks of age.

Standardized females (all the same age and same environmental conditions) were produced in addition to treatment individuals for use in reproduction assays.

Every two weeks, 150 eggs were collected and reared on a rich larval diet. Upon emergence, females were separated and housed in 2L containers (20 female flies per container) with sugar and yeast and water provided periodically. All standardized females were between two and three weeks post-emergence when used in reproduction assays.



**Fig. 1** Experimental design used to test for the effects of environment and age on male reproductive strategies. Eggs were randomly allocated to poor or rich larval diets and adult flies were randomly allocated to an adult diet and mating treatment combination in a fully crossed factorial design. Subsamples from each treatment combination were taken at 2, 4 and 6 weeks of age ( $n_{\text{total}}=96$ ).

#### Male reproductive performance assay

At ages 2, 4 and 6 weeks post adult emergence, a subsample of treatment males ( $n=4$  from each treatment combination) were transferred individually to a scintillation vial with a standardized virgin female for 6 hours in order to mate. This time period is long enough for mating to occur in nearly all pairs (EM, unpublished data). After mating, females were transferred to a 250ml container with oviposition medium (pre-moulded rich larval food) to allow egg laying.



Egg-output was counted after 72 hours 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 hours (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 100g of the standard poor larval diet (so that offspring larval environment was the same across treatments).

After 42 hours, the number of eggs to have hatched was recorded. Hatched eggs could be identified as empty egg-shells under a Leica M60 dissecting microscope (Leica Microsystems, Heerbrugg, Switzerland). Larvae were then left to develop in a standardized environment with the poor larval diet (previously shown to produce pronounced paternal effects (Bonduriansky and Head 2007)) at a constant temperature of 25°C with a light-dark cycle of 12 hours and watered periodically.

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

#### Male reproductive morphology

Each male's legs and wings were removed and a photo of the thorax was taken. The testes were then removed under a Leica M60 dissection microscope and photographed in a drop of PH buffering solution (enough to prevent desiccation but not enough to allow the testis to float) on a microscope slide. Photos were taken using a Leica MZ16A Stereoscope and a Leica DFC420 mounted camera with

standardised magnification. Measurements of testis area and thorax length were taken using Image J, version 1.47v (Rasband 2015).

### Statistical analysis

Egg-output and egg hatching success were analysed using a Generalised Linear Model with a “quasi-Poisson” correction for over-dispersion. Egg hatching success was analysed as count data with the number of eggs visible on the filter paper as a covariate (as 20 eggs were unable to be transferred for all replicates and not all the eggs transferred remained on the filter paper).

Egg-to-adult viability could not be analysed using a Poisson model due to zero-inflated data. It was therefore modelled as a binomial response where adult emergence was marked as 1 and non-emergence was marked as 0 for each egg transferred. This was analysed using Mixed Model Logistic Regression (individual as a random effect and all treatments and age interactions as fixed effects) and model reduction using AIC values and Likelihood ratio tests between reduced and previous model in which the reduced model was selected by removing the effect with the highest p-value using the anova function.

The effects of environment and age on testes area (standardised across the whole data set) was analysed using a General Linear Model. Thorax length (standardised within larval diet treatment because larval diet has a pronounced effect on body size) was included as a covariate to determine whether variation in testes area is explained by variation in body size within larval diet treatments. Testes area and thorax length (standardised within larval diet treatment) were included as covariates in all analyses of the response variables.

Some of the treatment males died, reducing the sample size at four and six weeks of age. To test if there were any treatment combinations that influenced survival with age, perhaps creating a selection effect, binomial data (survival versus death) for four and six week old males (two week old males were excluded as all except one male survived) was analysed using Logistic Regression with larval diet, adult diet, mating opportunity and age (4 or 6 weeks) as categorical, fixed effects. Logistic regression analysis indicated that probability of death did not differ among treatment combinations (see **Table S1**).

All analyses were completed using R, version 3.1.1 (R Development Core Team) with lme4 (Bates et al. 2014), MuMIn (Bartoń 2014) and car (Fox et al. 2014) packages used for the mixed-model logistic regression on egg-to-adult viability.

## Results

### Egg-output

Egg-output ranged from 0 to 125 eggs laid in the first 72 hours after mating (mean=36.85, SE=3.81). We detected a significant main effect of male age, an age × adult diet interaction, an age × mating × adult diet interaction and an age × mating × larval diet × adult diet interaction (**Table 1a; Fig. 2**). Because of the significant four-way interaction, the lower level interactions and the main effect of age must be interpreted with caution. Egg-output tended to increase from 2 to 4 weeks of age, with the exception of non-mated × poor larval diet × protein rich adult diet, in which egg-output was greatest at 2 weeks of age. In many treatment combinations,

egg output also declined from 4 to 6 weeks of age. Interestingly, the mated × rich larval diet × protein rich adult diet males had consistently low egg-output, whereas the non-mated × rich larval diet × protein poor adult diet males had consistently high egg output.

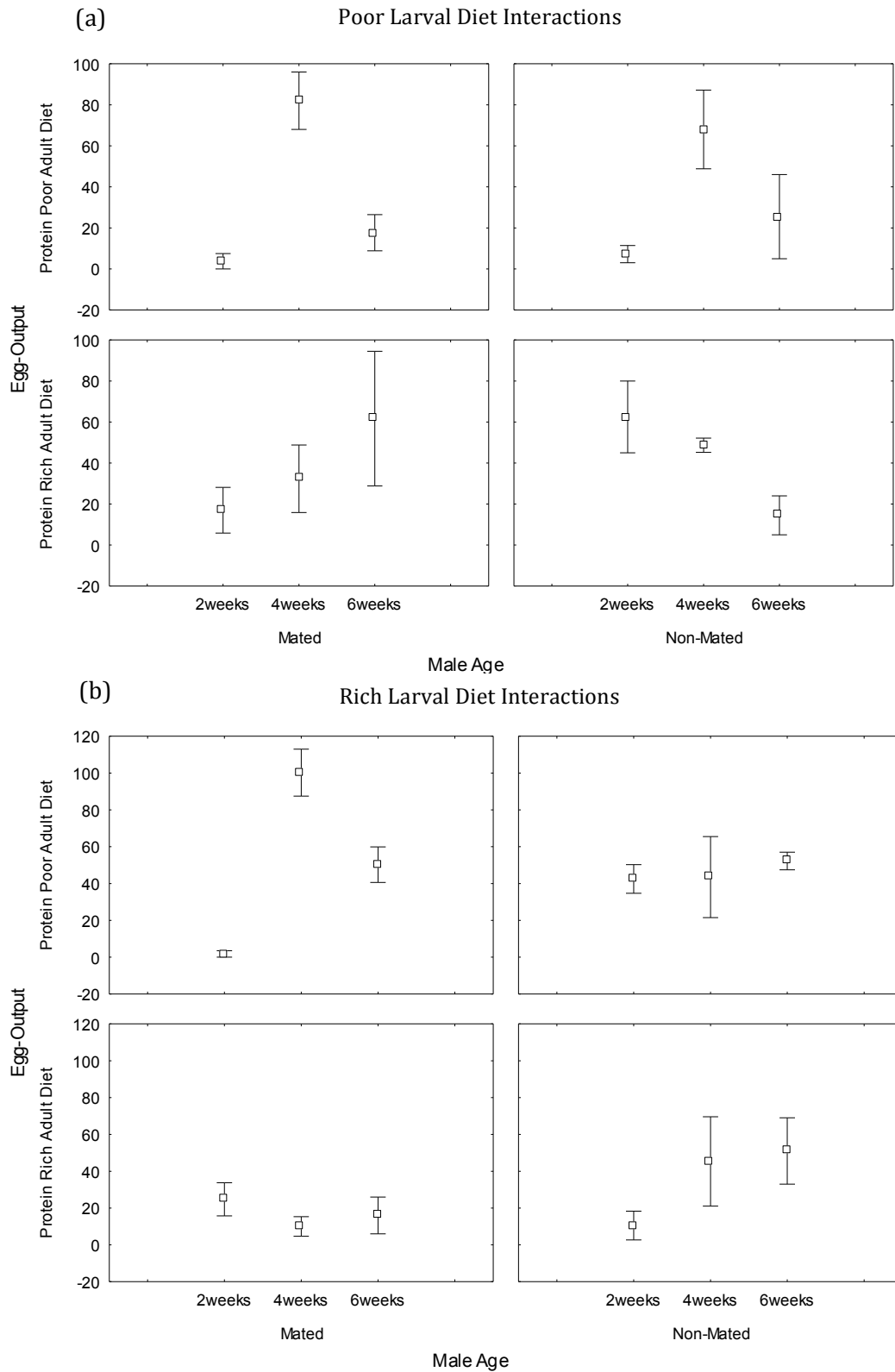
**Table 1**

The effects of male age, mating history, larval and adult diet with testis area and thorax length (standardised within larval diets) as covariates on egg-output (a), egg-hatching success (b), and egg-to-adult viability (c). For egg hatching success and egg-to-adult viability, simplified models are shown after excluding non-significant interactions (full models are shown in **Table S2**).

Predictor	Egg-Output <sup>a</sup>		Egg-Hatching Success <sup>b</sup>		Egg-to-Adult Viability <sup>c</sup>	
	Deviance Residual	P	Deviance Residual	P	Chi-Sq	P
Number of eggs visible	-	-	7.51	0.060	-	-
Testis area	13.61	0.346	0.2	0.757	0.54	0.463
Thorax length	19.80	0.256	0.2	0.759	1.96	0.162
Age	<b>508.40</b>	<b>&lt;0.001</b>	0.98	0.793	2.03	0.363
Mating	5.71	0.542	1.59	0.386	0.03	0.865
Larval diet	7.43	0.486	3.51	0.199	0.01	0.917
Adult diet	28.38	0.174	0.99	0.496	0.14	0.709
Age × adult diet	88.47	0.056	-	-	<b>10.85</b>	<b>0.004</b>
Age × larval diet	30.14	0.374	-	-	-	-
Age × mating	90.18	0.053	-	-	-	-
Mating × larval diet	1.36	0.766	-	-	-	-
Mating × adult diet	3.01	0.658	-	-	-	-
Larval diet × adult diet	<b>70.43</b>	<b>0.032</b>	-	-	-	-
Age × mating × larval diet	59.20	0.145	-	-	-	-
Age × mating × adult diet	209.26	0.001	-	-	-	-
Age × larval diet × adult diet	<b>106.74</b>	<b>0.031</b>	-	-	-	-
Mating × larval diet × adult diet	50.60	0.069	-	-	-	-
Age × mating × larval diet × adult diet	<b>136.16</b>	<b>0.011</b>	-	-	-	-

<sup>a</sup>Error DF=44

<sup>b</sup>Error DF=52



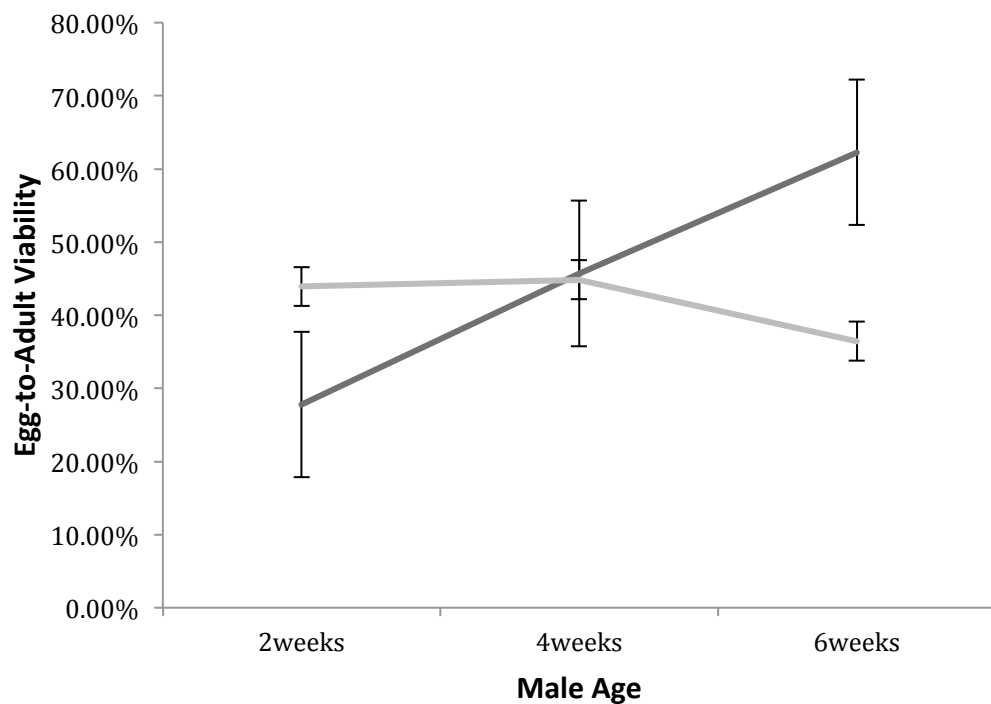
**Fig. 2** Effects of age, adult diet and mating opportunity on egg-output for males reared on a poor larval diet (a) and rich larval diet (b). The squares represent the mean and the bars represent standard errors.

### Egg hatching success

There were no significant interactions or main effects of the treatments and age on egg hatching success (**Table 1b; Table S2**). A mean of 79.67% (SE=3.67%) of the eggs hatched into larvae across all treatment and age combinations.

### Egg-to-adult viability

Egg-to-adult viability was significantly influenced by the interaction of male age and adult diet (**Table 1c; Fig. 3**). Egg-to-adult viability increased with advanced paternal age in males fed a protein rich adult diet, but decreased with advanced paternal age in males fed a protein poor adult diet.



**Fig. 3** Interaction of male age and adult diet on egg-to-adult viability. The dark-grey line represents the protein poor adult diet and the light-grey line represents the protein rich adult diet with the bars showing the standard errors.

### Testes area

Testes area was significantly influenced by an age  $\times$  mating, mating  $\times$  larval diet and an age  $\times$  adult diet interaction (**Table 2, Fig. 1S**). There was also a main effect of age, mating, larval diet and adult diet, although these effects must be interpreted with caution given the significant two-way interactions. Thorax length did not have a significant effect on testes area when included as a co-variate, indicating that testis size does not significantly change with a change in body size after accounting for the effect of larval diet.

**Table 2.**

General linear model for the effects of male age, mating history, larval diet and adult diet on testes size (standardised across the whole data set). Thorax length (standardised with larval diet) included as a covariate. The model was re-fitted after removing non-significant interactions (full model shown in **Table S3**).

Predictor	F	P
Age	<b>5.22</b>	<b>0.008</b>
Mating	<b>26.17</b>	<b>&lt;0.001</b>
Larval diet	<b>401.56</b>	<b>&lt;0.001</b>
Adult diet	<b>4.32</b>	<b>0.042</b>
Thorax size	0.92	0.3412
Age $\times$ mating	<b>3.87</b>	<b>0.026</b>
Mating $\times$ larval diet	<b>5.12</b>	<b>0.027</b>
Age $\times$ adult diet	<b>3.38</b>	<b>0.041</b>
Error DF=59		

Neither testes area nor thorax length had a significant effect on any of the response variables when included as covariates (**Table 1**).

## Discussion

We report age-dependent effects of male nutritional and mating environment on key components of male post-copulatory performance in *Telostylinus angusticollis*. A male's capacity to stimulate oviposition in their mates depends on a complex interaction of larval and adult diet, mating history and age. Although we found no male effects on egg hatching success, we observed an age-dependent paternal effect on offspring larval viability, mediated by protein content in the adult diet. We can infer that it is at the larval stage that paternal adult diet and age are influencing offspring viability as there were no effects on egg hatching success. This shows that male effects on female oviposition rate and offspring viability are affected in distinct ways by male environment and age.

Overall, a peak in egg-output at four weeks of age occurred in males that were fed a protein poor adult diet (with the exception of males fed a nutrient rich larval diet from the non-mated treatment in which egg-output remained the same with advanced age). Males with increasing egg-output with advanced age were fed a protein rich adult diet (with the exception of non-mated males from the nutrient poor larval diet that suffered a decrease in egg-output with advanced paternal age, and mated males from the nutrient rich larval diet that had consistently low egg-output). These effects demonstrate the complexity of the condition-dependent responses of egg-output.

The effects on egg-to-adult viability are much clearer, whereby male age and protein content in the adult diet interact together. Males fed a protein rich adult diet suffer reduced egg-to-adult viability with advanced paternal age compared to



protein poor males which have increasing egg-to-adult viability with advanced paternal age. Protein restriction theory predicts that individuals with a reduced protein intake experience an extended lifespan but with decreased reproduction due to a reallocation of limited resources to somatic maintenance (Boggs and Ross 1993, Partridge and Brand 2005). However, this is not the case here as males from the protein poor adult diet have higher larval-to-adult viability with advanced paternal age compared to males from the protein rich adult diet. Perhaps males from the protein rich diet are experiencing a greater cost of investment in egg-to-adult viability compared to that of males fed the protein poor adult diet and may be experiencing a form of toxicity from high protein intake (Simpson and Raubenheimer 2007).

A few experiments have shown a paternal effect on offspring viability in insects (Konior et al. 2001, Hosken et al. 2003, Garcia-Gonzalez and Simmons 2007, Simmons and Holley 2011) and in non-insect systems (Richard et al. 2005, Kekalainen et al. 2010, Firman and Simmons 2012). However, these primarily focus on the effects of polyandry and the influence of male genetic quality on the ability to sire viable offspring (Jennions and Petrie 2000, Simmons 2005). However, the significant interaction of paternal adult diet and age on offspring viability found in this study reflects an environment-dependent, non-genetic effect (see also Adler and Bonduriansky 2013). A study on *Drosophila melanogaster* demonstrated a reduction in larval viability due to large paternal body size with evidence suggesting that this was not due to male genetic quality. However, this was hypothesised to be due to sexually antagonistic effects, with females suffering a cost due to increased courtship and mating rate from larger males (Friberg and

Arnqvist 2003). This is unlikely in the current experiment as larval diet, which affects body size did not have a significant effect on egg-to-adult viability. Perhaps age and adult diet alter male courtship behaviour, how a concurrent study has demonstrated that male mating rate with a standard female in a short period of time does not alter reproductive output (chapter 2).

A study using the common lizard (*Lacerta vivipara*) has demonstrated male age effects on reproduction and offspring viability, with old males producing less viable offspring. This was hypothesised to be due to an accumulation of mutations during spermatogenesis (Richard et al. 2005). This cannot be the explanation for males fed the protein poor diet as egg-to-adult viability increases with age, however it is possible that males from the protein rich adult diet may accumulate mutations at a faster rate than that of males from the protein poor diet. This may then result in decreased larval viability. Protein-deprived males may be accumulating resources until nearing their maximal age and then invest heavily in reproduction in a form of terminal investment, compared to protein rich males that have the ability to invest highly at a young age but experience a cost to reproduction earlier in life. As there is no paternal provisioning in the form of a nuptial gift, it may be that these effects are mediated by SFPs.

A large array of SFPs have been found in the internally fertilising species studied (Eberhard and Cordero 1995, Chapman 2001, Pilch and Mann 2006, Perry et al. 2013), with greater than 100 Acps characterised in *Drosophila melanogaster* (Chapman 2001, Findlay et al. 2008) and a total of 923 different proteins found in human seminal fluid (Pilch and Mann 2006). Although not all functions are known, SFPs have been shown to alter a variety of physiological processes in females

(reviewed in Chapman 2001). Production of different SFPs are likely to impose differential costs for males, and these costs are expected to be reflected in condition dependent responses (Gillott 2003, Moore et al. 2004, Perry et al. 2013). The multiple SFPs that induce oviposition in the female in *Drosophila melanogaster* are likely to vary in degrees of plasticity and responses to environment and age. Although the SFPs produced by *Telostylinus angusticollis* have not yet been characterised, it is likely that they too produce multiple SFPs used in oviposition induction and this perhaps played a role in the complex interactions affecting egg-output.

Little is known about the SFPs influencing offspring viability, and this area requires much more investigation. However, proteins contained in the ejaculate have been shown to be incorporated into the female soma and ovarian tissues (Gwynne and Brown 1994, Rooney and Lewis 1999, Simmons 2011). SFP allocation to the female can then directly influence offspring viability due to absorption of proteins into the ovarian tissues or can alter female allocation to offspring due to the absorption of proteins into the female soma (Garcia-Gonzalez and Simmons 2005, Simmons 2011). There is also evidence in *Telostylinus angusticollis* to suggest that the paternal effects on offspring condition are transferred through components in the semen, rather than epigenetic changes to the DNA or female differential allocation (Crean et al. 2014). Although it is not known for certain that components in the semen are also conferring the paternal effect to larval viability, it can be inferred that SFPs may be mediating the age and adult diet effect on egg-to-adult viability and this is emphasised by the non

significant effect of testis size (used as proxy for sperm production) on any of the measures of male reproductive performance.

It is expected that female *T. angusticollis* fertilise their eggs by releasing sperm from the spermathecae as the eggs pass through the oviduct, like in other Diptera species (Hosken and Ward 1999, Arthur et al. 2008). Due to lack of significant effects of any of the variables on egg hatching success, egg hatching may not be mediated by males. This suggests that the effects on egg-to-adult viability are not due to differences in fertilisation success. This is also emphasised by the non-significant effects of testes size on any of the response variables, although testis size did change with male environment and age. This demonstrates that testis size is also subject to condition dependent effects, however testis size did not significantly influence any of the response variables and therefore the effects found on egg-output and egg-to-adult viability are not likely to be due to sperm production or allocation.

This is the first demonstration of a paternal effect on larval viability that is influenced by an interaction of dietary protein and male age. We also demonstrate the influence of multiple condition determining factors on male fecundity stimulation in the female. We suggest that the plasticity of egg-to-adult viability and egg-output are due to non-genetic effects through a mechanism such as SFPs. Testes area did not have an effect on any of the response variables, emphasising that the effects on reproduction are unlikely to be due to sperm production or allocation. We propose that the inclusion of multiple factors that can influence male condition gives a more accurate depiction of how they interact

together to influence male reproductive performance and that it is necessary to include trans-generational paternal effects such as offspring viability when measuring reproductive performance as this can also be subject to condition-dependent effects. Further investigation into a mechanism driving these responses would be beneficial.

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## Appendix

**Table s1**

Full model for the effects of male age, mating history, larval diet and adult diet on the survival of the treatment males using binomial logistic regression. There were no main effects found in the additive model.

Predictor	F	P
Age	1.2	0.279
Mating	0.3	0.586
Larval Diet	1.2	0.279
Adult Diet	2.7	0.107
Age × mating	0.3	0.586
Age × larval diet	0.0	1.000
Mating × larval diet	0.3	0.586
Age × adult diet	0.3	0.586
Mating × adult diet	0.0	1.000
Larval × adult diet	2.7	0.107
Age × mating × larval diet	0.3	0.586
Age × mating × adult diet	0	1.000
Age × larval × adult diet	0.3	0.586
Mating × larval × adult diet	0.0	1.000
Age × mating × larval diet × adult diet	0.0	1.000
Error	DF=48	

**Table s2**

Full model for the effects of male age, mating history, larval and adult diet on egg hatching success using a generalised linear model with a “quasi-Poisson” correction for over-dispersion. The number of eggs visible on the filter paper, testis area and thorax length included as covariates.

Predictor	Deviance	
	residual	P
Number of eggs visible	7.507	0.103
Testis area	0.203	0.789
Thorax length	0.201	0.790
Age	0.983	0.840
Mating	1.593	0.453
Larval Diet	3.506	0.265
Adult Diet	0.985	0.555
Age × mating	1.930	0.711
Age × larval diet	1.484	0.769
Mating × larval diet	3.892	0.241
Age × adult diet	6.420	0.321
Mating × adult diet	0.023	0.928
Larval × adult diet	5.656	0.157
Age × mating × larval diet	6.797	0.300
Age × mating × adult diet	0.028	0.995
Age × larval × adult diet	0.248	0.957
Mating × larval × adult diet	0.058	0.886
Age × mating × larval diet × adult diet	8.164	0.236

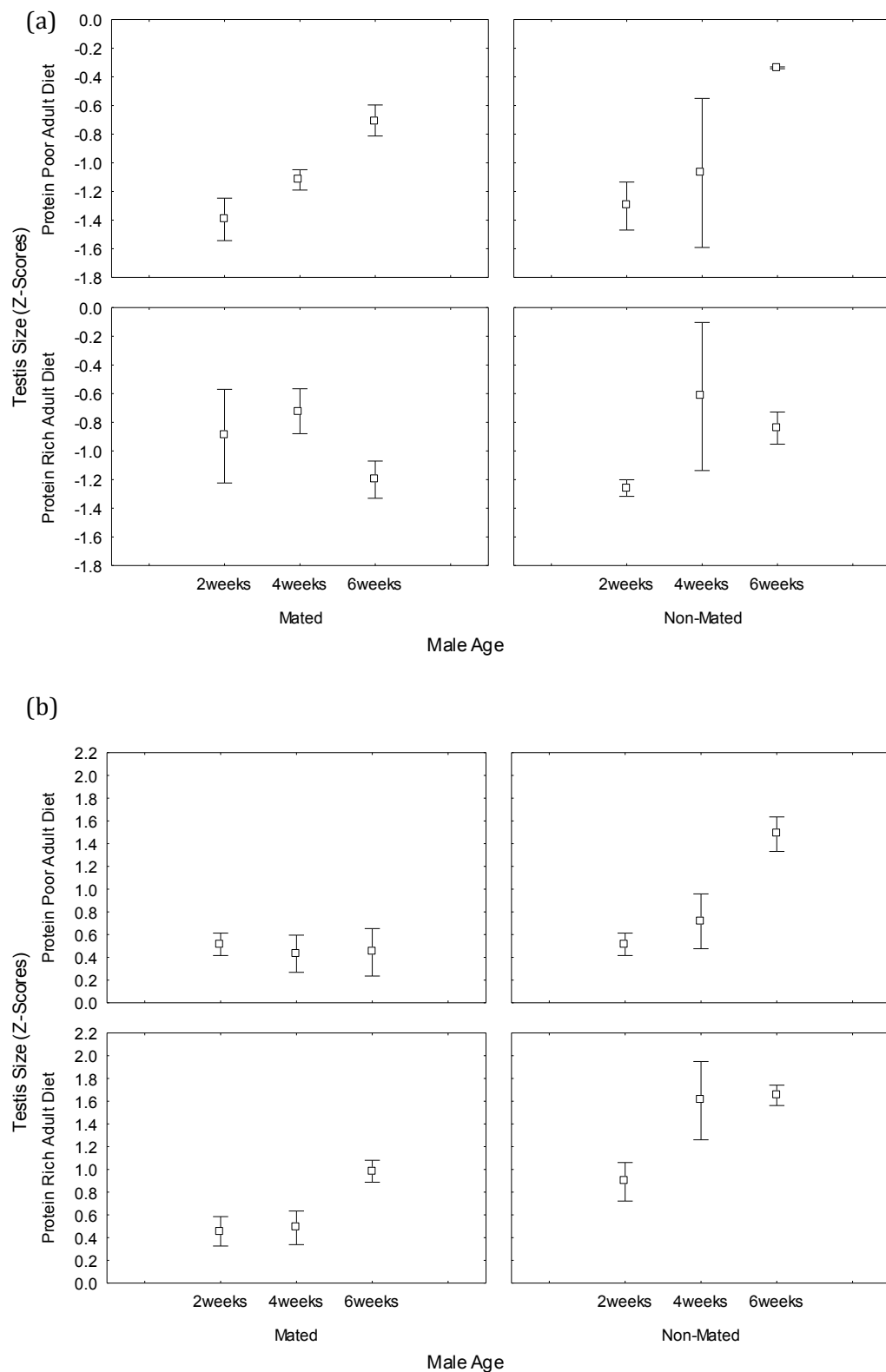
Standard error=3.67%, Error DF=34

**Table s3**

Full model for the effects of male age, mating history, larval and adult diet on testis size (standardised across the whole data set) using a general linear model. Thorax size (standardised with larval diet) was included as a covariate.

Predictor	F	P
Age	<b>5.343</b>	<b>0.008</b>
Mating	<b>26.770</b>	<b>0.000</b>
Larval Diet	<b>410.803</b>	<b>0.000</b>
Adult Diet	<b>4.424</b>	<b>0.041</b>
Thorax length	0.940	0.337
Age × mating	<b>3.961</b>	<b>0.026</b>
Age × larval diet	0.793	0.459
Mating × larval diet	<b>5.146</b>	<b>0.028</b>
Age × adult diet	<b>3.014</b>	<b>0.059</b>
Mating × adult diet	0.541	0.466
Larval × adult diet	1.694	0.200
Age × mating × larval diet	0.278	0.759
Age × mating × adult diet	0.965	0.388
Age × larval × adult diet	2.462	0.097
Mating × larval × adult diet	1.586	0.214
Age × mating × larval diet × adult diet	1.258	0.294





**Fig. s1** Effects of age, adult diet and mating opportunity on male testis size (standardised across the whole data set) for males reared on a poor larval diet (a) and rich larval diet (b). The squares represent the mean and the bars represent standard errors.

## CHAPTER TWO

### **An investigation of the direct and trans-generational costs of ejaculate depletion in *Telostylinus angusticollis***

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ELM, AC and RB conceived and designed the experiment, ELM carried out the experiment, analysed the data and wrote the manuscript, AC and RB provided supervision and guidance throughout the experiment, analysis and writing process.

## Abstract

Males can be limited by the number of ejaculates that can be produced, as well as the quality of ejaculate transferred. This may be costly not only due to the possibility of decreased fertilisation potential, but due to the decrease of components found in the semen such as seminal fluid proteins (SFPs) that can stimulate oviposition and influence paternal effects. It is known that frequent mating can reduce the number of progeny sired by the male, however it is not known if frequent mating can also decrease paternal effects. In this study, we investigate the consequences of ejaculate depletion in the neriid fly, *Telostylinus angusticollis*. Males were either allowed to mate frequently over 6 hours (mated treatment) or exposed to females but prevented from mating (non-mated treatment), and then paired with a standardised virgin female to quantify male mating rate, reproductive output and paternal effects on offspring viability and condition. Although males from the mated treatment exhibited reduced mating rate following the treatment period, there was little evidence of a cost of previous mating in terms of reproductive output or paternal effects. However, mating treatment affected the rate of reduction in male accessory gland size with each subsequent mating, and accessory gland size affected larval viability. These results indicate that males of *T. angusticollis* do not suffer an obvious cost to frequent mating on reproductive performance. However, these results suggest that frequent mating depletes the accessory glands, with potential consequences for offspring viability.

## Introduction

Theory predicts that male reproductive success should increase with increased insemination of females (Bateman 1948). However, because of the cost of ejaculate production, males are often limited in the number of times they can mate (Dewsbury 1982). Males can transfer sperm packed in spermatophores or seminal nuptial gifts that are either absorbed into the female oocytes or the female soma (Vahed 1998) – significantly increasing the cost of ejaculate production and making them subject to depletion (Dewsbury 1982, Wedell et al. 2002, Parker and Pizzari 2010). It was first observed by Halliday and Houston (1978) in a newt species, *Triturus vulgaris* and by Rutowski (1979) in a butterfly species, *Pieris protodice*, that males were limited by the production of spermatophores. In *T. vulgaris*, male courtship intensity was limited by the number of spermatophores produced and in *P. protodice*, spermatophore size decreased with increased mating number, demonstrating that males may either be limited in the number of ejaculates produced or limited by ejaculate size/quality. Since then, this pattern has been observed numerous times across many taxa including species where males transfer no spermatophore or nuptial gift (reviewed in South and Lewis, 2011). These effects are likely due to the many proteins and peptides contained within the ejaculate that are important for male reproductive success but increase the cost of production (Eberhard and Cordero 1995, Chapman 2001, Simmons 2001, Moore et al. 2004, Poiani 2006, Avila et al. 2011).

Seminal fluid proteins (SFPs), mainly synthesised in the accessory glands (Wolfner 1997, Chapman 2001, Perry et al. 2013) are a highly studied component of the

male ejaculate. In *Drosophila melanogaster*, over 100 proteins have been characterised (Chapman 2001, Findlay et al. 2008) and a total of 923 different proteins have been found in human seminal fluid (Pilch and Mann 2006). There are many functions of SFPs that are found across taxa that can aid sperm transfer and fertilisation and can increase male paternity share in polyandrous species (Leopold 1976, Poiani 2006). SFPs can alter female physiology such as increasing ovulation, egg-laying, latency to re-mate and sperm storage (Chapman 2001, Gillott 2003, Poiani 2006), all of which are important for males faced with sperm competition. There is much evidence to suggest that these can be depleted due to frequent mating and that mating number may be limited by the male's ability to replenish SFPs. For example, frequent mating results in smaller ejaculate size (Rutowski 1979, Dewsbury 1982, Wedell et al. 2002, Torres-Vila and Jennions 2005, Marcotte et al. 2007, South and Lewis 2011, Michaud et al. 2013), and this then results in a corresponding decrease in male reproductive output (Lefevre and Jonsson 1962, Hihara 1981, Linklater et al. 2007, Sirot et al. 2009, Garcia-Gonzalez and Simmons 2010). It has been demonstrated in *Drosophila melanogaster*, the stalk eyed fly (*Cyrtodiopsis dalmanni*) and the bed bug (*Cimex lectularius*) that frequent mating significantly decreases the size of the accessory glands compared to the testes (Rogers et al. 2005, Linklater et al. 2007, Reinhardt et al. 2011), indicating that SFPs are depleted at a faster rate compared to sperm. It has also been shown that between three and five matings are enough to decrease accessory gland size, egg-output and the number of progeny produced in *Drosophila melanogaster*, demonstrating how quickly depletion can occur (Hihara 1981, Linklater et al. 2007). Therefore, ejaculate depletion due to frequent mating can reduce a male's ability to fertilise eggs and stimulate oviposition.

Males can also influence offspring phenotype and viability through non-genetic paternal effects (reviewed in Crean and Bonduriansky 2014). Although there are many mechanisms through which this can occur, evidence in some species suggests that this can occur through components in the semen such as SFPs (Michaud et al. 2013, Crean et al. 2014, Mirhosseini et al. 2014). For example, males of two holometabolous Diptera species, *Telostylinus angusticollis* and *Drosophila melanogaster*, have been shown to influence the body condition of their offspring through a non-genetic paternal effect, with high condition fathers reared on nutrient rich larval diets producing high condition offspring (Bonduriansky and Head 2007, Valtonen et al. 2012, Adler and Bonduriansky 2013). Moreover, research by Crean et al. (2014) demonstrated that the paternal effect on offspring condition found in *T. angusticollis* is likely transmitted through components of the semen rather than epigenetic changes to the DNA in the sperm. This may be due to proteins transferred by the male that are absorbed directly into the developing eggs, or proteins may be incorporated into the female soma influencing female allocation to offspring (Simmons 2011, Crean et al. 2014). Paternal larval diet and social environment (Adler and Bonduriansky 2013) as well as paternal age and adult diet (chapter 1) have also been shown to influence paternal effects on larval viability in *T. angusticollis* and it is likely that these effects may also be due to components in the semen.

There is much research indicating a cost to frequent mating in males due to a decrease in ejaculate components that results in a decrease in progeny sired (Linklater et al. 2007, Reinhardt et al. 2009, Sirot et al. 2009, Perry and Rowe 2010, Reinhardt et al. 2011, Michaud et al. 2013, Perry and Tse 2013). However, there is

little research examining trans-generational costs, whereby frequent mating decreases a male's ability to induce condition-dependent paternal effects in his offspring. Some paternal effects, such as increased offspring body size and increased offspring viability are fitness-enhancing for the male and therefore males should be selected to confer these paternal effects to as many offspring as possible. However, this may make them subject to depletion if the mechanism that confers these effects is costly. The study by Michaud et al. (2013) is one of the few to investigate an effect of frequent mating on paternal effects using two *Coccinella* species. It was found that larval survival in the first instar significantly decreased and development time increased after frequent mating in *Coccinella transversalis*, and a similar but weaker pattern was found in *Coccinella septempunctata*. It was also found that male mating history decreased female offspring weight in *Cheilomenes sexmaculata* (Mirhosseini et al. 2014). However, this area of research requires a lot more investigation as the few studies examining trans-generational effects due to male mating history have all been conducted on Coccinellid beetles and it is important to test such effects in other groups. If male ejaculate components are depleted due to frequent mating, it could be expected that any condition-dependent trans-generational effects previously observed should decrease.

Here, we aimed to examine the costs of mating for males of *Telostylinus angusticollis* by experimentally depleting the components of the ejaculate that influence male reproductive output as well as the trans-generational paternal effects on offspring viability and phenotype. Mating history was manipulated by

enabling some males to mate frequently in a short period of time while exposing other males to females but preventing them from mating. Males were then mated to standard virgin females. Treatment effects on the number of matings with the standard females in the period following the treatment were quantified, as well as egg-output, egg hatching success, and paternal effects on offspring viability and offspring body size. Treatment effects on male testis size (used as a proxy for sperm number) and accessory gland size (used as a proxy for SFPs) were also examined as well as effects of male testis and accessory gland size on male reproductive performance. We predicted that both egg-output and the paternal effects on offspring viability and offspring phenotype would decrease after frequent mating and that there would also be a corresponding decrease in accessory gland size. Previous work suggested that egg hatching success is not plastic and is not an SFP-induced trait but rather a function of sperm transferred/fertilisation success (chapter 1). Hence, if sperm is also depleted, there may be a decrease in egg hatching success and testis size.

## **Materials and methods**

### Study species

*Telostylinus angusticollis* is found on the east coast of Australia where they mate and feed on rotting bark. Females are polyandrous and can store sperm for approximately two weeks (EM, unpublished data), exposing males to sperm competition. Males transfer no spermatophore that is thought to contain any nourishment for the female; rather, sperm are transferred in bundles (groups of spermatids held together in parallel) prior to copulation and the spermatids



dissociate after insemination (EM, unpublished data). 25 male and 25 female wild flies were collected from Keep Reserve, Castlecrag, Sydney (33.80° S, 151.22° E) and maintained in laboratory population cages using standard rearing protocols (Bonduriansky and Head 2007) over two generations prior to the experiment.

#### Rearing of experimental flies

2500 eggs were collected from the stock flies and all larvae were reared on a standard nutrient rich diet consisting of coco-peat, molasses, liquid barley malt and protein powder (as described in Bonduriansky, 2007). This diet was selected as Bonduriansky and Head (2007) demonstrated that high condition males reared on the standard rich diet produce high condition offspring and therefore are good candidates to examine a decrease in condition-dependent paternal effects. 150g of larval food per 50 eggs (50 larval containers) was considered enough to control for effects of larval density on emerging adult body size (EM, unpublished data).

Larval containers were kept at a constant temperature of 25°C with a 12 hour light-dark cycle and watered periodically.

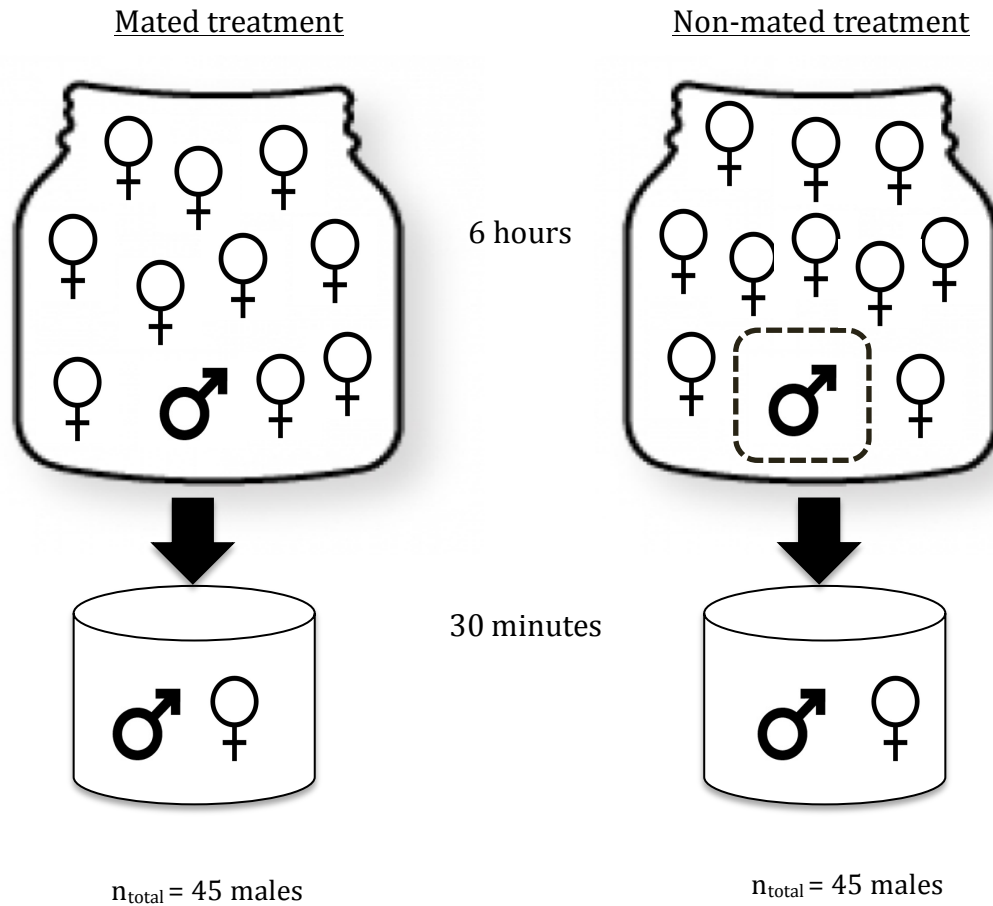
After adult emergence from the 2500 eggs transferred to larval medium, males were immediately relocated to 250ml containers where they were housed individually until three weeks old and then they were randomly paired with a second male for one week ( $n_{\text{total}}=90$  individual males). Males were paired because males kept individually may not transfer any sperm during the first mating (EM, unpublished data). Adult males were fed brown sugar but not provided with protein in order to reduce the opportunity for replenishment of depleted ejaculate reserves.

Females to be used as standard virgin females were randomly collected after emergence and housed in groups of 20 in 2L plastic containers. The remaining females to be used in the treatments were randomly allocated into groups of 10 in 1.5L plastic containers where the males were placed during the treatments. All females and males were four weeks of age and virgins when placed in treatments. All enclosures for both males and females contained damp cocopeat (watered periodically to provide hydration), a petri dish with brown sugar for the males and brown sugar and yeast for the females, and were covered with a stocking to allow ventilation.

#### Male mating assay

From each pair, one randomly selected male was allocated to the mated treatment and the other male was allocated to the non-mated treatment. Males in the mated treatment ( $n_{\text{total}}=45$  males) were each placed in a 1.5L container with 10 virgin females. The number of times the males mated in the first hour was recorded. Each mating takes approximately 45 seconds and observations during the first hour of this study showed that males mated up to 15 times (mean=8.67, SD=1.290, Range=2–15). Most males were still observed mating after six hours. Males in the non-mated treatment ( $n_{\text{total}}=45$ ) were kept in individual scintillation vials with a mesh lid and placed in the centre of a 1.5L container housing 10 virgin females. This allowed the males to see the females as well as receive pheromone cues through the mesh, but prevented mating. All males were kept in the treatments for 6 hours and provided with water but no food. After the 6 hour treatment, each male was paired with a standard virgin female in a scintillation vial for 30 minutes

(**Fig. 1**) and the number of times each male mated with the standard female was recorded.



**Fig. 1** Male mating assay: males were housed in a mated or non-mated treatment for 6 hours and then paired with a standard virgin female for 30 minutes.

Males were then frozen at  $-80^{\circ}\text{C}$  for later dissection. The standard females were placed individually in new 250ml containers with oviposition medium (pre-moulded rich larval food).

Egg-output was recorded after 96 hours and a random sample of 20 eggs were collected from each standard female to measure egg hatching success, egg-to-adult viability and offspring body size. If no eggs were laid by 96h, egg output was recorded as 0, but eggs laid after 96h were used to quantify other response

variables. If less than 20 eggs were laid, eggs were checked daily until 20 eggs could be used to quantify the other response variables. Eggs were placed on damp filter paper (to facilitate quantification of egg hatching success) and the filter paper was placed on top of 100g of poor larval diet (a three-fold reduction in nutrients compared to that of the standard rich larval diet that the parent flies were raised on). Previous research suggests that paternal effects are most pronounced in flies reared on the poor larval diet (Bonduriansky and Head (2007). After 42 hours, the number of eggs that had hatched was recorded (previous observations show that this is enough time to allow all viable eggs to hatch (EM, unpublished data)). Hatched eggs could be identified as empty shells under a Leica M60 dissecting microscope (Leica Microsystems, Heerbrugg, Switzerland). After eggs were collected, females were frozen at -20°C for measurement of body size.

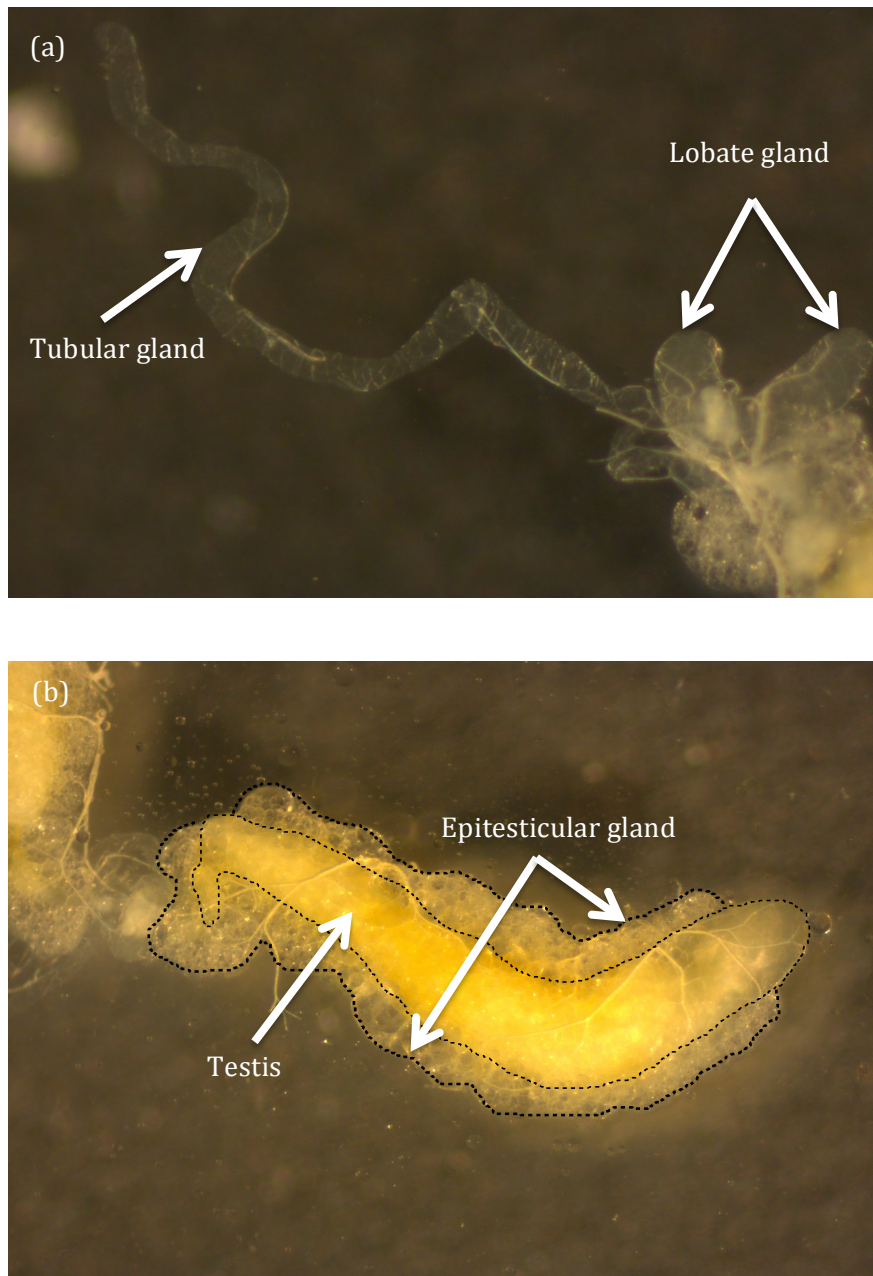
Each larval container was maintained at 25°C with a light-dark cycle of 12hrs and watered periodically until pupation. After 20 days, individual larval containers were then placed inside individual 2L plastic containers for the offspring to emerge into. Egg-to-adult viability was measured as the number of adult flies to emerge per treatment male after two weeks from the first emergence.

#### Morphometric data

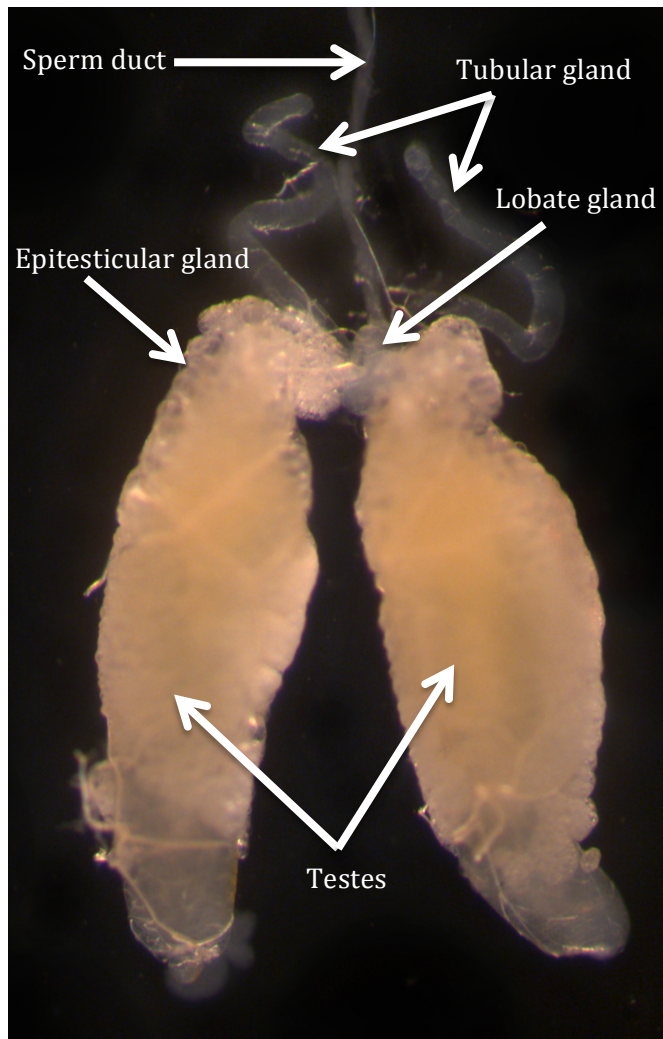
After emergence, offspring were frozen and a random sample of five males and five females (where possible) per treatment male were randomly selected to measure offspring body size. Offspring and maternal and paternal thorax length were measured from photos taken under standardised magnification after removing legs and wings. Paternal testes and accessory glands were removed and photographed on a slide moistened with PH buffering solution (enough to prevent

desiccation but not enough to allow the testis to float). All dissections were carried out under a Zeiss (Stemi 2000-CS) microscope (Zeiss, Jena, Germany) and photos were taken using a mounted Zeiss AxioCamERc5s camera under standardised magnification. Measurements of thorax length, testes and accessory gland areas were calculated using Image J, version 1.47v (Rasband 2015).

The sizes of three structures that appeared to be accessory glands were quantified. One accessory gland structure is long and tubular (“tubular gland”), while another is sac-like (“lobate gland”) (**Fig. 2 (a)**). Each of these structures consists of two lobes. The sizes of these structures were quantified by measuring the area of one of the lobes of the tubular gland and the total area of both lobes of the lobate gland. The size of the glandular tissue surrounding the testes (“epitesticular gland”) (**Fig. 2 (b)**) was quantified as the thickness of the glandular tissue, calculated as the difference between the total area of the glandular tissue and testis and the area of the testis. One testis and epitesticular gland was calculated per male. The location of the three accessory glands relative to the testes and sperm duct is shown in **Fig. 3**.



**Fig. 2** Images of the three putative accessory glands and testis of *Telostylinus angusticollis*.



**Fig. 3** Image of the internal reproductive structures of *Telostylinus angusticollis*.

### Statistical analysis

The effects of treatment on the number of matings with the standard female, egg-output, egg hatching success and egg-to-adult viability were analysed using Generalized Linear Models with a Poisson distribution ( $X\beta = \ln(\mu)$ ) and corrected for over-dispersion using the “quasi-Poisson” procedure. Effects were tested using Wald statistics. Because not all egg shells remained on the filter paper after 42 hours, the number of eggs visible on the filter paper was included as a covariate in the analysis of egg hatching success. Egg hatching success was included as a

covariate for the number of offspring to emerge as adults, therefore indicating the viability of the larval and pupal stage after the eggs had hatched (larval-to-adult viability). The number of times the males mated with the standard females following the treatment, and paternal and maternal body size were included as covariates for egg output, egg hatching success and larval-to-adult viability. The number of times the males mated during the first hour of the treatment was included as a covariate for the number of times the males mated with the standard female in the 30 minutes following the treatment. Male testis and accessory gland sizes were also included as covariates in egg-output, egg hatching success and larval-to-adult viability models.

Treatment effects on offspring body size were analysed using a General Linear Model with maternal and paternal body size, the number of times the males mated with the standard female and male testis and accessory gland sizes included as covariates, and offspring sex included as a within-subjects factor. There was no within-subjects effect of offspring sex so the mean body size for each brood was used in the model. There was also no interaction of maternal or paternal body size, so this was removed from the model.

The effects of treatment and the number of matings with the standard female on male testis and accessory gland size were analysed using a General Linear Model.

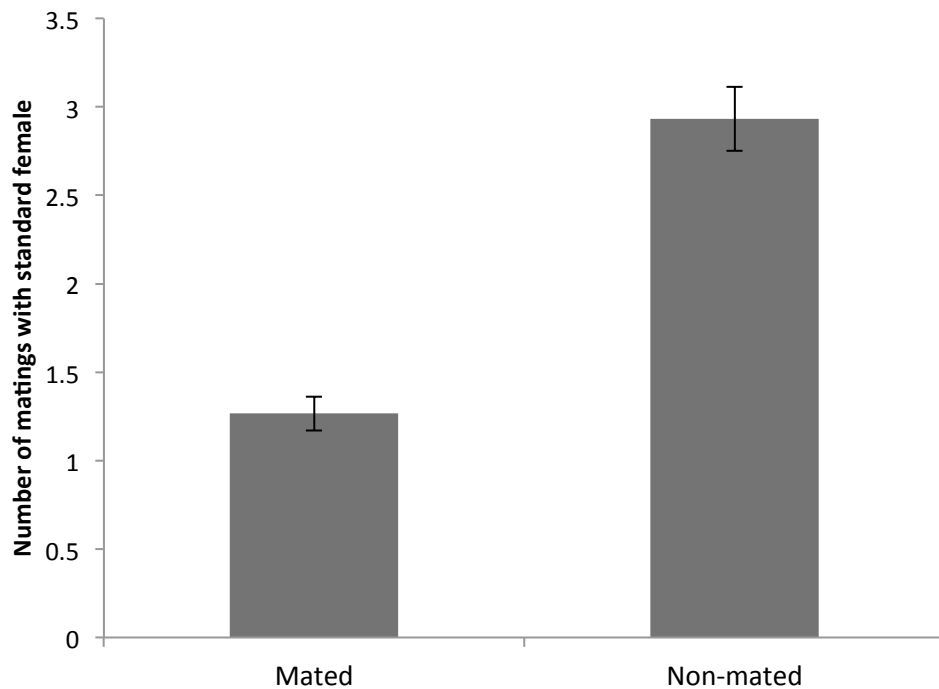
All analyses were completed using Statistica (release 7, Statsoft, Tulsa, Oklahoma, USA).



## Results

### Mating rate

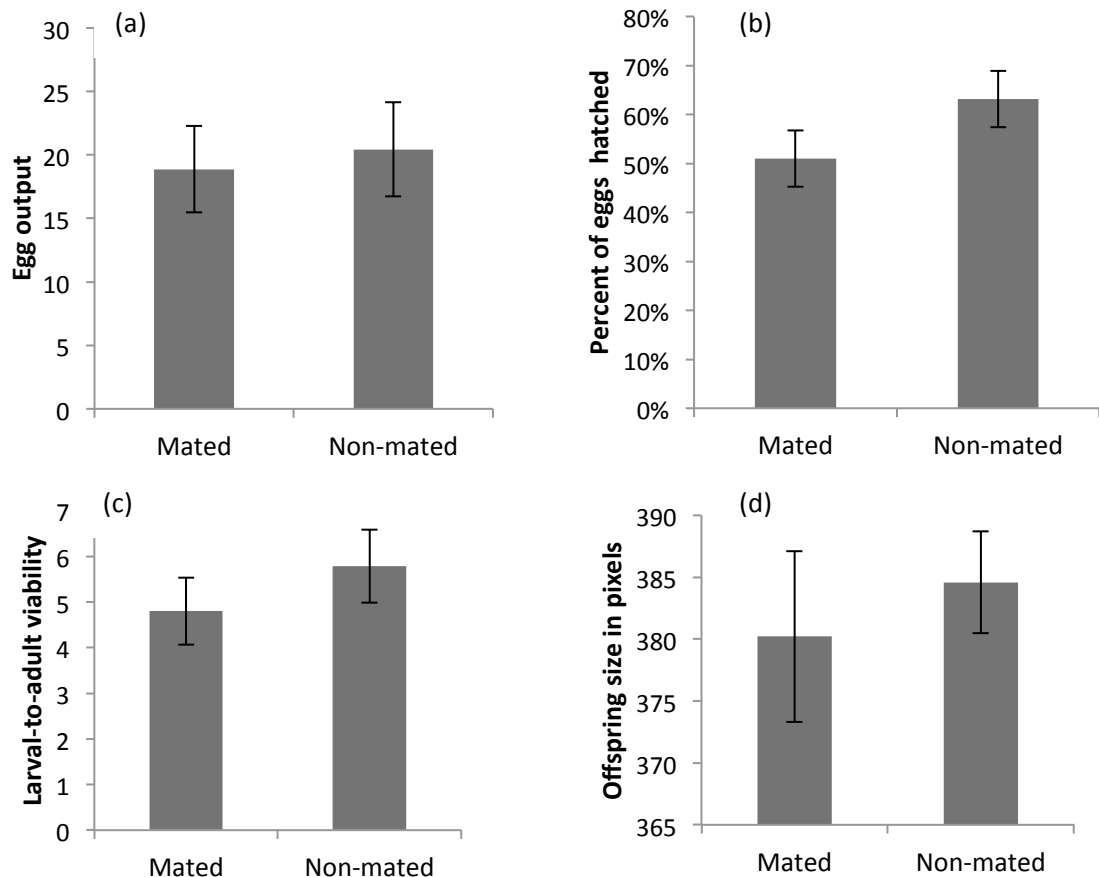
Males from the mated treatment mated significantly fewer times with the standard females compared to males from the non-mated treatment (Wald stat=16.27, DF=1,  $p < 0.001$ ; **Fig. 4**). The number of times the males were observed mating during the first hour of the treatment did not have an effect on the number of times they mated with the standard females (Wald stat=0.03, DF=1,  $p = 0.8525$ ).



**Fig. 4** Mean number of matings with the standard females for males from the mated treatment and the non-mated treatment with standard error shown by the error bars.

### Male reproductive output and paternal effects

There was no significant effect of treatment or the number of times the males mated with the standard females on egg-output, egg hatching success, larval-to-adult viability or offspring body size (**Fig. 5; Table 1; Table 2**).



**Fig. 5** The effects of treatment (mated or non-mated) on egg-output (a), egg hatching success (b), larval-to-adult viability(c) and offspring body size (d). The error bars show standard errors.

The size of the lobate glands had a significant effect on larval-to-adult viability, with larger lobate accessory glands associated with higher viability (**Table 1**).

There was no significant effect of testis size or the three accessory gland sizes on egg output, egg hatching success (**Table 1**) or offspring body size (**Table 2**).

**Table 1**

Effects of treatment (mated versus non-mated), mating number with the standard female, maternal and paternal body size and male reproductive morphology on egg output (a), egg hatching success (b), and larval-to-adult viability (c) with the number of eggs visible on the filter paper as a covariate for egg hatching success and egg hatching success included as a covariate for larval-to-adult viability.

Predictor	Egg output <sup>a</sup>		Egg hatching success <sup>b</sup>		Larval-to-adult viability <sup>c</sup>	
	Effect	p	Effect	p	Effect	p
Intercept	<b>3.133</b>	<b>&lt;0.00</b>	0.298	0.969	0.214	0.763
Treatment	-0.213	0.226	-0.169	0.523	-0.070	0.455
Number of eggs visible	-	-	0.121	0.751	-	-
Egg hatching success	-	-	-	-	<b>0.123</b>	<b>&lt;0.000</b>
Mating number with	0.037	0.906	-0.225	0.418	-0.151	0.613
Maternal body size	<b>0.699</b>	<b>0.029</b>	0.345	0.145	-0.017	0.944
Paternal body size	0.479	0.456	-0.128	0.802	-0.210	0.633
Testis size	0.233	0.380	-0.044	0.826	0.097	0.595
Tubular glands	0.107	0.627	0.113	0.517	-0.042	0.797
Lobate glands	-0.349	0.299	-0.074	0.751	<b>0.369</b>	<b>0.039</b>
Epitesticular glands	0.003	0.994	-0.323	0.145	0.363	0.182

**Table 2**

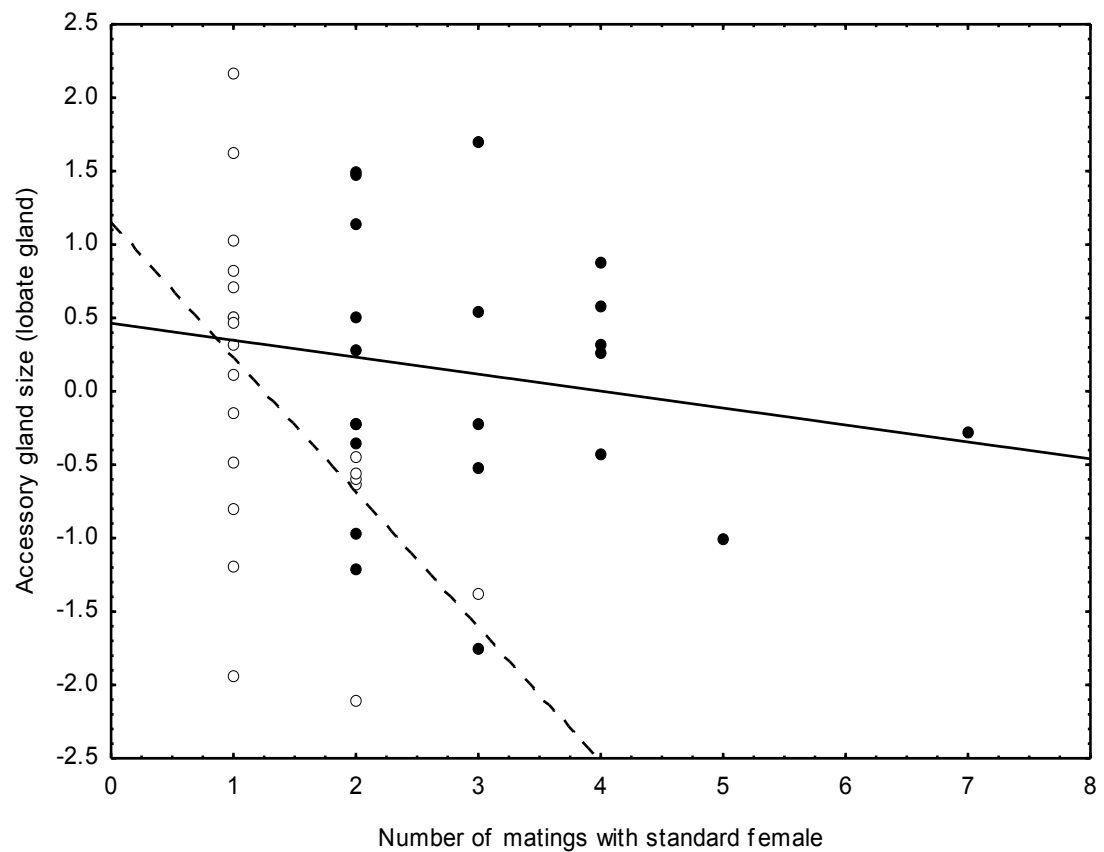
Effects of treatment (mated versus non-mated) on offspring body size, with maternal and paternal body size, the number of matings with the standard female and male reproductive morphology as covariates.

Predictor	F statistic	P-value
Intercept	0.171	0.850
Treatment	0.294	0.765
Maternal body size	1.080	0.443
Paternal body size	4.319	0.131
Number of matings with standard female	0.373	0.717
Testis size	0.274	0.778
Tubular gland	0.635	0.589
Lobate gland	0.508	0.646
Epitesticular gland	2.395	0.239

#### Effects of mating treatment on male reproductive morphology

The size of the lobate glands was affected by the number of matings with the standard female, as well as the interaction of number of matings with the standard female and mating treatment (**Table 3; Fig. 6**): the size of the lobate glands declined more rapidly with number of matings for males from the mated treatment compared to males from the non-mated treatment. However, there was no main effect or interaction found for treatment or the number of times the male mated

with the standard female on testis size, or the size of the tubular glands or the epitesticular glands (**Table 3**).



**Fig. 6** Scatter plot showing the interaction between the number of times the males mated with the standard females and treatment (mated versus non-mated) on the size of the lobate glands (open circles and dotted line = mated treatment, closed circles and solid line = non-mated treatment).

**Table 3** Effects of treatment and the number of times the male mated with the standard female on testis size (a), and the three accessory glands (b,c,d).

	Testis <sup>a</sup>		Tubular gland <sup>b</sup>		Lobate gland <sup>c</sup>		Epitesticular gland <sup>d</sup>	
Predictor	F value	p	F value	p	F value	p	F value	p
Intercept	0.338	0.563	0.031	0.861	<b>4.535</b>	<b>0.040</b>	0.508	0.478
Treatment	1.089	0.300	0.395	0.534	0.822	0.370	0.833	0.364
Matings with standard female	0.717	0.400	0.087	0.770	<b>6.962</b>	<b>0.012</b>	1.097	0.298
Treatment X Matings with standard female	0.673	0.414	0.055	0.816	<b>4.199</b>	<b>0.047</b>	0.996	0.321

## Discussion

We report that males of *Telostylinus angusticollis* change their mating behaviour after mating for 6 hours, with males from the mated treatment mating significantly fewer times after the treatment period compared to males from the non-mated treatment. However, frequent mating had no significant effect on the males' ability to induce oviposition, egg hatching or on offspring viability or size. This suggests that the costs of mating on the reproductive performance that directly follows a period of high mating opportunity are low for males of this species, and that neither the components of the ejaculate, such as seminal fluid proteins (SFPs) or

sperm approached full depletion after six hours of continuous mating opportunity. This is surprising, as costs of frequent mating in males have been well documented in other species (Halliday and Houston 1978, Rutowski 1979, Hihara 1981, Linklater et al. 2007, Perry and Rowe 2010, Reinhardt et al. 2011, Michaud et al. 2013, Perry and Tse 2013). The high condition dependence of male reproductive output and paternal effects previously found in *T. angusticollis* are also indicative of an allocation of limited resources, suggesting a cost to investing in multiple facets of reproductive performance (chapter 1, Bonduriansky and Head 2007, Adler and Bonduriansky 2013, Adler et al. 2013). However, contrary to our predictions, it was found that frequent mating had little effect on male reproductive performance in this species.

There was no significant effect of treatment on any of the reproductive traits measured, however it is possible that costs of mating may become apparent with additional frequent matings, or that significant treatment effects would have been observed with a larger sample size. However, these results suggest that males of this species can achieve a high number of matings and suffer little cost compared to that of other insect species (Hihara 1981, Rogers et al. 2005, Linklater et al. 2007, Reinhardt et al. 2011, Michaud et al. 2013). Males of *Drosophila melanogaster* need only three to five matings over five hours to reduce the size of accessory glands and reduce the number of progeny sired (Hihara 1981, Linklater et al. 2007), and males of *Coccinella transversalis* only need five matings over five days to have a trans-generational effect on offspring development time and first instar survival (Michaud et al. 2013). The males of *T. angusticollis* were observed mating an average number of 8.67 times in the first hour of the treatment period

and a majority were still observed mating at the end of the six hours. Therefore, it is interesting that males of *T. angusticollis* were able to mate at such a high rate compared to *D. melanogaster* and *C. transversalis* and yet achieve similar reproductive performance to virgin males.

It is possible that there may be more latent costs that were not detected in this study, such as decreased longevity due to an allocation of resources to reproduction rather than somatic maintenance. One model presented by Cameron et al. (2007) suggests that male reproduction may not be limited by ejaculate investment but males may suffer a reduction in lifespan. The idea of a trade-off between reproduction and lifespan is commonly used when examining costs of mating for females. However, there is evidence to suggest that mating may also reduce the lifespan of males (Spratt 1980, Partridge and Farquhar 1981, Partridge and Andrews 1985, Perry and Tse 2013). In the Coccinellid, *Adalia bipunctata*, some extreme costs to mating have been observed, including a four-fold reduction in lifespan (Perry and Tse 2013). However, less pronounced but significant reductions to lifespan have been observed in males of other species (reviewed in Scharf et al. 2013). Adler et al. (2013) examined the effects of nutrients in the larval diet, adult diet and social environment on male reproduction and lifespan in *Telostylinus angusticollis*, and found that changes in male lifespan and reproduction depended on a particular environmental combination and did not provide support for an obvious lifespan-reproduction tradeoff. Therefore, it is not clear if males of this species suffer lifespan costs due to mating environment and it would be beneficial to examine this further.



We detected an interaction of treatment and the number of times males mated with the standard females on the size of the lobate accessory glands, with males from the mated treatment suffering a steeper decline in accessory gland size with each additional mating compared to males from the non-mated treatment. This suggests that there may be a loss of accessory gland products such as SFPs with each mating, and that this loss may be relatively greater in males with a recent history of frequent mating. Males with smaller accessory glands also conferred lower larval-to-adult viability on their offspring, suggesting that the products of these glands may influence offspring viability in a dose-dependent manner. Thus, male mating history may indirectly influence offspring viability by altering the rate at which the accessory glands are depleted. However, there is a wide range of accessory gland sizes for males from the mated treatment that only mated once with the standard female which therefore will result in a wide range of larval viabilities, and this may have prevented the detection of a treatment effect on larval viability. This aspect of a potential cost to mating for males requires further investigation, however this is one of the few studies that demonstrates a trans-generational effect of male accessory gland size on offspring viability (likely due SFPs) and shows the potential for these to be depleted with frequent mating.

In summary, these results suggest that males of *Telostylinus angusticollis* are able to mate frequently, with numbers that far exceed those of previously studied insect species (Hihara 1981, Reinhardt et al. 2011, Michaud et al. 2013, Perry and Tse 2013), and yet suffer little or no observed reduction in reproductive output or paternal effects on offspring viability and condition. This suggests that perhaps a cost of frequent mating on future male reproductive performance may not be as

common as the literature suggests, and this idea should be tested across as many study systems as possible in order to provide a broader understanding of the costs of mating for males. We also found that larval viability is influenced by paternal accessory gland size (likely due to the amount of seminal fluid proteins transferred) and that accessory gland size decreases at a faster rate with mating number for males that had been previously mated compared to that of virgin males. This suggests the possibility for a trans-generational effect on larval viability that may be influenced by male mating history and we propose that future studies should include trans-generational paternal effects as a measure of male reproductive performance. We also suggest that while there may not be an obvious cost on male reproductive performance, there may be more latent costs such a decrease in longevity. Future studies should also include effects on male lifespan and responses under different environmental conditions including varying nutrients in the diet to determine if this accentuates any costs that may be present.

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## General Conclusion

This thesis demonstrates the effects of multiple condition-determining factors on male reproductive performance using the neriid fly, *Telostylinus angusticollis*.

In chapter one, it was found that larval viability of offspring is influenced by the amount of protein found in the paternal adult male's diet and that this changed with advanced paternal age. Males that had been fed a high protein adult diet suffered a reduction in larval viability of the offspring with advanced paternal age, whereas males that had been fed a low protein diet had increasing larval viability of the offspring with advanced paternal age. It was also demonstrated that male-induced egg-output is influenced by an interaction of nutrients consumed at the larval and adult stage, male mating history and male age. There was no effect of male age, larval and adult diet or mating history on the number of eggs to hatch in to larvae, indicating that in natural environments, the number of larvae produced and the viability of the larvae is likely to vary greatly with environment and that paternal protein consumption and paternal age are key drivers of this.

While the mechanism driving the responses found in chapter one is not known for certain, it is suggested that these effects may be due to components found in the semen such as seminal fluid proteins (SFPs). SFPs have been shown to have a wide range of influences on female physiology including male-induced egg-output (Wolfner 1997, Chapman 2001, Perry et al. 2013) and potentially offspring viability (Simmons 2011). It was found that testes size did not influence egg output, egg hatching or egg-to-adult viability, suggesting that sperm

production/allocation did not have an effect on male reproductive performance in *T. angusticollis*; further supporting the idea that SFPs may have been driving the responses. This was later supported in chapter two where it was shown that males with larger accessory glands (where many seminal fluid proteins are produced) produce offspring with higher larval viability.

Surprisingly, chapter two demonstrated that males of *T. angusticollis* suffer no large cost to frequent mating on male reproductive performance, with males from the mated treatment achieving nearly the same level of reproductive performance as that of virgin males, although there was a decrease in mating rate. This is surprising given that much of the theoretical and empirical literature suggests that male reproductive performance should decrease with increased mating rate (Halliday and Houston 1978, Rutowski 1979, Hihara 1981, Dewsbury 1982, Parker and Ball 2005, Linklater et al. 2007, Reinhardt et al. 2011, Michaud et al. 2013). *T. angusticollis* males showed no significant decrease in the number or quality of offspring produced after mating frequently over a period of 6 hours, suggesting that male reproductive output in *T. angusticollis* is more consistent with Bateman's original theory that male reproductive output is limited by access to females rather than by resources (Bateman 1948).

Although no effect of mating treatment on male reproductive performance was detected, males with larger accessory glands produced offspring that were more likely to survive to the adult stage. Furthermore, males from the mated treatment suffered a steeper decline in accessory gland size with mating number compared to non-mated males, indicating that perhaps there is the potential for a trans-generational effect of male mating history that is mediated by seminal protein

stores in the accessory glands. However, an effect of male mating history on larval viability was not found and therefore no conclusive statement can be made on whether there is a trans-generational cost to larval viability.

Overall, this thesis demonstrates the importance of male environment male and condition as sources of variation in reproductive performance, including trans-generational paternal effects. It demonstrates that protein consumed by the male at the adult stage and male age interact together to influence the viability of the offspring and that male-induced egg-output is highly plastic to multiple condition-determining variables. It is also demonstrated that a cost of mating on future male reproductive performance is not ubiquitous across all species. Both chapters demonstrate an influence of males on offspring larval viability and this is likely conferred through components in the semen such as seminal fluid proteins. The plasticity of trans-generational paternal effects is not taken into account in much of the literature and I demonstrate the need to examine trans-generational effects such as offspring viability when investigating the plasticity of male reproductive performance as these can also be susceptible to condition dependent responses. Therefore, this thesis presents interesting and novel insights into the plasticity of male post-copulatory reproductive performance and demonstrates the importance of including measures of trans-generational paternal effects as a measure of male reproductive performance.

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