Variation in male post-copulatory investment: ontogeny to progeny

ERIN L MACARTNEY

VARIATION IN MALE POST-COPULATORY INVESTMENT:

ONTOGENY TO PROGENY

A thesis in fulfilment of the requirements for the degree of
Doctor of Philosophy

September 2019

School of Biological, Earth and Environmental Sciences
Faculty of Science

THESIS SHEET

Surname/Family Name	:	Macartney
Given Name/s	:	Erin Larissa
Abbreviation for degree as give in the University calendar	:	PhD
Faculty	:	Science
School	:	School of Biological, Earth, and Environmental Sciences
Thesis Title	:	Variation in male post-copulatory investment: ontogeny to progeny

Condition-dependence theory has been widely applied to exaggerated male signaling traits, and more recently to certain sperm and semen traits. However, such post-copulatory traits can be highly cryptic and multi-faceted, and the published literature shows considerable variation in trait expression due to male condition. This thesis aims to uncover sources of variation in post-copulatory trait expression, and to incorporate male investment in ejaculate and offspring quality into a condition-dependence life-history framework. Using meta-analyses and meta-regression in Chapter Two, I demonstrate that much of the variation in post-copulatory responses to nutrient limitation (used as a manipulation of condition) is accounted for by differences in the type of nutrients, the ontogenetic life-stage when nutrients are limited, and the type of trait. Trait responses are also taxon-specific. In Chapter Three, I empirically demonstrate that developmental nutrient limitation strongly reduces testes and accessory gland size, as well as sperm movement within the female reproductive tract, and that adult diet does not alter such responses in the neriid fly, *Telostylinus angusticollis.* In Chapters Four and Five, I consider condition-dependent effects on offspring quality. In Chapter Four, I argue that non-genetic paternal effects conferred through epigenetic factors may also be costly, condition-dependent traits. In Chapter Five, I use *T. angusticollis* to test if frequent mating results in a condition-dependent reduction in fecundity and offspring quality. Surprisingly, frequent mating did not result in reduced fecundity or offspring quality, but did result in a reduced mating rate. Finally, in Chapter Six, I demonstrate that male *Drosophila melanogaster* suffer sperm depletion across successive matings, but the rate of depletion is not dependent on diet or genotype. Instead, males vary in ability to mate multiply, and individuals that mate more also transfer more sperm. Thus, variation in male post-copulatory performance depends substantially on unknown factors that are unrelated to nutrition and genotype. Overall, this thesis demonstrates that sperm and semen traits are affected by many different factors, and that variation in such traits can be highly complicated. Understanding differences in post-copulatory trait expression will provide increased understanding into the evolution of mating systems and reproductive investment.

FOR OFFICE USE ONLY Date of completion of requirements for Award:

ORIGINALITY STATEMENT

'I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.'

Sign.. …….....

20/09/2019

Date …….....

INCLUSION OF PUBLICATIONS STATEMENT

UNSW is supportive of candidates publishing their research results during their candidature as detailed in the UNSW Thesis Examination Procedure.

Publications can be used in their thesis in lieu of a Chapter if:

- The student contributed greater than 50% of the content in the publication and is the "primary author", i.e., the student was responsible primarily for the planning, execution and preparation of the work for publication
- The student has approval to include the publication in their thesis in lieu of a Chapter from their supervisor and Postgraduate Coordinator.
- The publication is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in the thesis

Please indicate whether this thesis contains published material or not.

☐ *This thesis contains no publications, either published or submitted for publication*

☒ *Some of the work described in this thesis has been published and it has been documented in the relevant Chapters with acknowledgement*

☐ *This thesis has publications (either published or submitted for publication) incorporated into it in lieu of a chapter and the details are presented below*

CANDIDATE'S DECLARATION

I declare that:

- I have complied with the Thesis Examination Procedure
- where I have used a publication in lieu of a Chapter, the listed publication(s) below meet(s) the requirements to be included in the thesis.

Name	Signature	Date (dd/mm/yy)
Erin L Macartney		10/01/2020

PUBLICATIONS, PRESENTATIONS AND AWARDS

Publications

Macartney, E.L., Crean A. J., Nakagawa, S., Bonduriansky, R. (2019). Effects of nutrient limitation on sperm and seminal fluid: A systematic review and meta-analysis. *Biological Reviews*: 94, 1722—1739.

Macartney E.L., Nicovich R. R., Bonduriansky R., Crean A. J. (2018). Developmental diet irreversibly shapes male post-copulatory traits in the neriid fly (*Telostylinus angusticollis*). *Journal of Evolutionary Biology:* 31, 1894–1902.

Macartney E.L., Crean A.J., Bonduriansky R. (2018). Epigenetic paternal effects as costly, condition-dependent traits. *Hereditary:* 121, 248–256.

Macartney E.L., Crean A.J., Bonduriansky R. (2017). Adult dietary protein has age- and context-dependent effects on male post-copulatory performance. *Journal of Evolutionary Biology*: 38, 42–49.

Nicovich, P.R., **Macartney, E.L**., Whan, R.M. and Crean, A.J. (2015). Measuring sperm movement within the female reproductive tract using Fourier analysis. *Microscopy and Microanalysis*: 21, 256–263.

Presentations

UNSW 3-Minute Thesis Competition, Sydney, Australia. (2018). You are what you eat: dietary effects on male reproduction.

Science Faculty Research Show Case, Sydney, Australia. (2018). You are what you eat: dietary effects on male reproduction – first place.

Australasian Evolutionary Society conference, Hobart, Australia. (2017). Condition-dependent post-copulatory traits: a meta-analysis.

Biology of Spermatozoa Conference, Bakewell, United Kingdom. (2017). Cryptic non-genetic paternal effects as condition-dependent traits.

European Evolutionary Society conference, Groningen, Netherlands. (2017). Cryptic non-genetic paternal effects as condition-dependent traits.

Behaviour conference, Cairns, Australia. (2015). No cost of mating for male neriid flies?

International Society for Behavioural Ecology conference, New York, USA. (2014). No cost of mating for male neriid flies?

Australasian Evolution Society conference, Geelong, Australia. (2013). Effects of age, diet, and mating on male post-copulatory performance in the neriid fly.

Awards

Australasian Evolution Society Student Award (2019)

European Society of Evolutionary Biology Godfrey Hewitt Mobility Award (2019)

Faculty of Science Post Graduate Research Competition: First Place (2018)

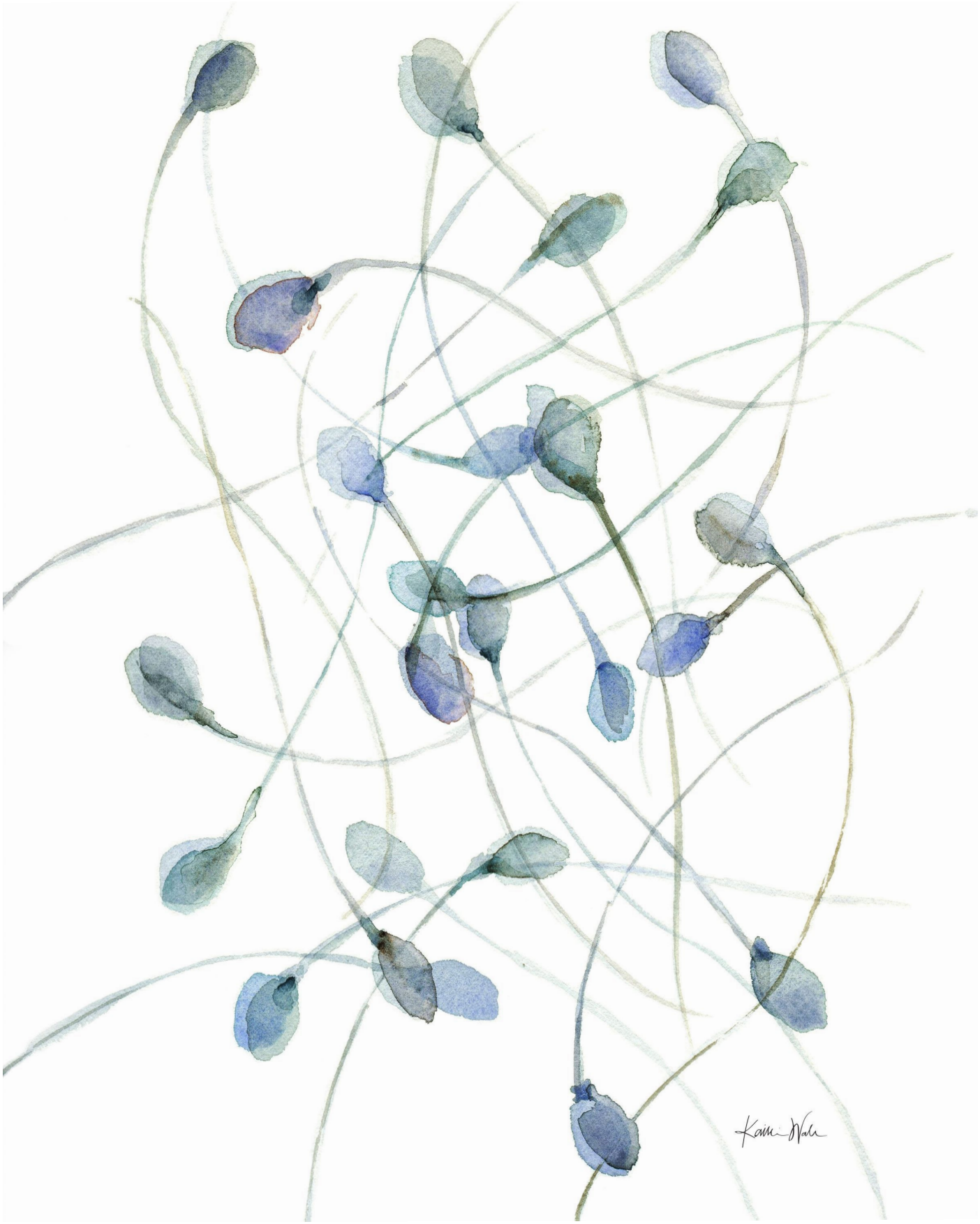
Australasian Evolution Society Student Award (2017)

Australian Postgraduate Research Award (2016)

Australasian Evolution Society Student Award (2015)

"Of the branches of biological science to which Charles Darwin's life-work has given us the key, few, if any, are as attractive as the subject of Sexual Selection"

R.A. Fisher, 1915



P LRA

CONTENTS

ACKNOWLEDGEMENTS

I would like to dedicate this thesis to my grandad, Robert Macartney. His passion for science and teaching provided me with an interest in the natural world from a young age.

This thesis would not have been possible without the support and dedication of my supervisor, Russell Bonduriansky. When I moved to Sydney to complete a Masters, I didn't imagine that I would be here nearly seven years later and close to finishing a PhD. Throughout this time, he has provided endless support, and has allowed me to explore and hone my own scientific interests. I am inspired by his passion for pure science, and he has taught me many valuable skills that I will take into the future. For this and much more, I am incredibly grateful.

Throughout my post-graduate career, Ange Crean has also been a huge source of support. She has been a fantastic role model as a woman in science, and I really value all the advice and time she has given me. I am also highly grateful to all the other collaborators throughout my PhD; in particular, Shinichi Nakagawa and Stefan Lüpold. Both Shinichi and Stefan patiently helped me develop new skills that were very daunting at first. I feel very lucky to have had the pleasure of working with such amazing researchers.

I have also had the pleasure of being part of a fantastic research centre and lab group. The Evolution and Ecology Research Centre is filled with so many inspiring and supportive researchers that have provided such a sense of community. In particular, Rob Brooks and Steve Bonser for being on my panel, Dan Noble for helping with the Post-Graduate Committee, Dan Falster for organising Friday seminars, Terry Ord for hiring me as a research assistant, and Mike Kasumovic for always being the life of the office. I would also like to thank the past and present members of the Bond Lab, including Nathan, Amy, Zac, Felix, Margo, and Ange. Having such a supportive lab group has really enriched my time at UNSW and I can't wait to see where we all end up in the future! Also, a big thanks to Jono and Vera for providing such fantastic support to the E&ERC.

There have been so many post-grad students that have made my time as a PhD student memorable. A special shout-out to the beans of BagSoc – Stanky (Stewie), Shriv (Dax), Flickn' (Chris G), Peeled (Chris S), Jumpin' (Amy), and the newest members, Green (Tiff), Keen (Fonti), and Stalky (Francesca). You guys have made my PhD so enjoyable! There have been so many spontaneous nights at Cook House and Bat Country breaking down nitty gritty topics of life (like how to give a good handshake and when to hug people) that were the cause of many days in bed watching Netflix and eating pizza the next day. But, these memories (or lack thereof) are what gives a PhD a sense of community and a source of support, and I wouldn't trade them for the world.

I was also lucky enough to temporarily become part of a new research centre whilst visiting the Lüpold Lab at the University of Zürich. A huge thank you to Valérian, Jhoniel, Alessio, Abhishek, Manvi, Sonja, Jeannine, and of course Stefan. I had the best time with you all (even though you call me Erin McChicken), and I laughed so much! Also, thank you to Tash for making me feel at home and always being up for an adventure, and a big thank you to Christoph, Marianne, and Anna Kopps for welcoming me into your home, and for all the long chats over white wine spritzers. I was made to feel so welcome during my time in Switzerland and have so many fond memories that I will cherish forever.

Outside of UNSW, the support of very close, long-time friends provided me with a respite from science when I needed it. A very special thank you to Damon and Brett, for always being there when I need it. Thank you to Nick for always showing interest in my research, and to Hannah for answering all my grammar and font related questions. Also, a big thank you to Cheye and Kelly for being such amazing housemates during my PhD and for all the fun nights at the CBH. These friendships, along with others, provided me with a great work-life balance and I am very grateful for your perpetual support.

Also, thank you to my Mum for giving me the freedom to pursue any path I have wanted in life, and for graciously accepting that I have chosen financial instability for the last ten years. I appreciate it.

Lastly, thank you to all the Neriids and Drosophila that unwillingly gave their lives to the research in this thesis. Your sacrifice has made a generous contribution to our understanding of reproductive plasticity.

Apologies to anyone not named in this thesis. There have been so many people that have made my PhD journey very special and I am very thankful to anyone that was a part of it!

ABSTRACT

Condition-dependence theory has been widely applied to exaggerated male signaling traits, and more recently to certain sperm and semen traits. However, such post-copulatory traits can be highly cryptic and multi-faceted, and the published literature shows considerable variation in trait expression due to male condition. This thesis aims to uncover sources of variation in post-copulatory trait expression, and to incorporate male investment in ejaculate and offspring quality into a condition-dependence life-history framework. Using meta-analyses and meta-regression in Chapter Two, I demonstrate that much of the variation in post-copulatory responses to nutrient limitation (used as a manipulation of condition) is accounted for by differences in the type of nutrients, the ontogenetic life-stage when nutrients are limited, and the type of trait. Trait responses are also taxon-specific. In Chapter Three, I empirically demonstrate that developmental nutrient limitation strongly reduces testes and accessory gland size, as well as sperm movement within the female reproductive tract, and that adult diet does not alter such responses in the neriid fly, *Telostylinus angusticollis*. In Chapters Four and Five, I consider condition-dependent effects on offspring quality. In Chapter Four, I argue that non-genetic paternal effects conferred through epigenetic factors may also be costly, condition-dependent traits. In Chapter Five, I use *T. angusticollis* to test if frequent mating results in a condition-dependent reduction in fecundity and offspring quality. Surprisingly, frequent mating did not result in reduced fecundity or offspring quality, but did result in a reduced mating rate. Finally, in Chapter Six, I demonstrate that male *Drosophila melanogaster* suffer sperm depletion across successive matings, but the rate of depletion is not dependent on diet or genotype. Instead, males vary in ability to mate multiply, and individuals that mate more also transfer more sperm. Thus, variation in male post-copulatory performance depends substantially on unknown factors that are unrelated to nutrition and genotype. Overall, this thesis demonstrates that sperm and semen traits are affected by many different factors, and that variation in such traits can be highly complicated. Understanding differences in post-copulatory trait expression will provide increased understanding into the evolution of mating systems and reproductive investment.

LIST OF FIGURES

Chapter Two

Chapter Four

Nil.

Chapter Five

Chapter Six

LIST OF TABLES

Chapter Two

Chapter Three

Chapter One

GENERAL INTRODUCTION

Erin L Macartney

The vast diversity of sexual traits within and between species has sparked immense interest in biologists and naturalists since Darwin and Wallace. Much of this interest has been aroused by the clear exaggeration and variation of male secondary sexual traits such as ornaments and weaponry, and understanding the evolutionary processes that generate such exaggeration and variation has become a prominent research field in evolutionary biology (see Darwin, 1859, 1871, Wallace, 1889; Fisher, 1915, 1930; Zahavi, 1975).

Central to sexual selection theory is the idea that genetic diversity alone cannot account for the large amount of variation in sexual traits within species (Rowe & Houle, 1996). This is because sexual traits can vary substantially with environmental factors that can determine male condition (i.e., the pool of resources available for investment in costly life-history traits) (Andersson, 1982; Nur & Hasson, 1984; Grafen, 1990; Rowe & Houle, 1996). Persistent directional selection leads to greater trait exaggeration and increases the energetic cost of producing and maintaining such traits. Therefore, individuals in good condition can invest more in costly sexual traits compared to individuals in poor condition – making sexual trait expression strongly condition-dependent (e.g., Moller and Delope, 1994; Emlen, 1997; Kotiaho, 2000; Judge *et al.*, 2008). However, it is not just the striking secondary sexual traits that show such exaggeration and variation. Antonie Van Leeuwenhoek first described diversity in spermatozoa in 1677, and while selection on sperm traits was not recognised until the latter-half of the 20th century (Parker, 1970), there is evidence that sperm and seminal fluid traits can be subject to similar evolutionary processes as secondary sexual traits. It is the variation in these post-copulatory traits that interests me, and that this thesis is concerned with.

Contrary to Trivers's (1972) assumption that male investment in sperm is energetically cheap, studies have now shown that selection can act to increase sperm and seminal fluid traits in the same way it acts to increase the size of secondary sexual traits (e.g., Linklater *et al.*, 2007; Rowe *et al.*, 2015; Lüpold *et al.*, 2016; Godwin *et al.*, 2017). Investment in such traits can be costly (e.g., Dewsbury, 1982; Olsson *et al.*, 1997; Perry and Tse, 2013; Godwin *et al.*, 2017) and condition-dependent (e.g., Droney, 1998; Kahrl and Cox, 2015; Kaldun

and Otti, 2016; Wigby *et al.*, 2016). However, there are many research questions that remain to be addressed. In particular, the published responses of male post-copulatory traits to differences in male condition are highly variable in strength and direction. This variation in trait responses is likely, in part, due to the cryptic and highly multifaceted nature of sperm and seminal fluid traits. Also, many empirical studies examining post-copulatory condition-dependence vary in several biologically relevant variables. For example, male condition is often manipulated by varying a range of nutritional components such as total calories, protein, or micronutrients, and this manipulation is applied at different ontogenetic life-stages. Thus, there is compelling need to distinguish underlying sources of variation that alter condition-dependent trait expression. Additionally, due to the multi-faceted nature of post-copulatory sexual selection, there are other post-copulatory traits that have not yet been incorporated into a condition-dependence life-history framework such as factors within the sperm or seminal fluid that can mediate non-genetic paternal effects. In this thesis, I address sources of variation in post-copulatory investment, and consider male investment in offspring quality through 'cryptic' non-genetic factors (i.e., epigenetic factors and small amounts of proteins and peptides).

Prior to my PhD, no quantitative synthesis of the post-copulatory condition-dependence literature had been attempted. Consequently, studies are often cited selectively, and broad patterns are difficult to discern. In Chapter Two, I used the powerful tool of meta-analysis and meta-regression to quantify the variation in post-copulatory trait responses to nutrient limitation (used as a manipulation of male condition) and examined underlying sources of variation in trait responses. After sorting through over 2100 studies from my initial literature search, I compiled 348 effects of nutrient limitation on sperm and seminal fluid traits from 71 papers and 50 species of arthropods and vertebrates that met my stringent selection criteria. I also collected information on the type of nutrient manipulation (i.e., calories, protein, fats, and micronutrients), the type of post-copulatory trait (i.e., sperm quantity, movement, morphology, and seminal fluid quantity), the ontogenetic life-stage when nutrients were limited (i.e., during development or in adults), and the

broad taxonomic group (i.e., fish, arthropods, and mammals). Through this, I show that there is indeed a very high amount of variation in published responses of sperm and seminal fluid traits to nutrient limitation and a substantial proportion of this variation can be attributed to differences among response traits, taxa, and experimental treatments. I hope that this chapter will provide interesting new insights into how male post-copulatory traits respond to nutrient availability, and will highlight new questions regarding the environments that generated selection for certain patterns of post-copulatory condition-dependence. Ultimately, such follow-up studies will help to illuminate how differences in ejaculate trait expression correspond to differences in fitness.

While compiling the database of the published post-copulatory responses to nutrient limitation for Chapter Two, it became apparent that there were no studies that comprehensively examined how nutrient limitation at different ontogenetic life-stages (i.e., during development or as adults) interact to affect post-copulatory condition-dependence. Chapter Two demonstrated that both developmental and adult nutrient availability are important for post-copulatory traits in many taxonomic groups. However, it was evident that few studies had examined whether adult nutrient availability altered effects of developmental nutrient availability (but see Amitin & Pitnick, 2007; Vermeulen, Engels, & Sauer, 2008; Melo *et al.*, 2014). Determining how nutrients at the different life-stages interact is crucial for understanding when investment in post-copulatory traits is determined, and if the nutrient availability at one life-stage can alter the effects of nutrient availability at the other – resulting in even greater variation in post-copulatory trait expression compared to if post-copulatory plasticity was limited to discrete life-stages. For example, nutrient limitation at both life-stages could result in a stronger 'cumulative' condition-dependent response compared to if nutrients are only limited at one life-stage, or a nutrient rich adult diet could compensate for the condition-dependent effects of developmental nutrient limitation.

In Chapter Three, I used a fully-crossed experimental design of nutrient-rich and nutrient-poor diets at the developmental (larval) and adult stages of a

native Australian fly species, *Telostylinus angusticollis* (Neriidae) to examine how dietary nutrients at different life-stages interact to affect male post-copulatory trait expression, including testes and accessory gland size, sperm length, and sperm movement within the female reproductive tract. Pre-copulatory traits important for male-male combat and monopolising habitat patches containing females are strongly condition-dependent due to larval nutrient availability in this species (Bonduriansky, 2007; Sentinella, Crean, & Bonduriansky, 2013), but there is also likely to be strong selection on post-copulatory trait expression due to the high risk of sperm competition. Therefore, due to the previously demonstrated sexual-trait plasticity and potential strong directional selection on post-copulatory ejaculate traits, *T. angusticollis* makes an ideal study system to examine dietary effects on post-copulatory trait expression. It was predicted that male investment in and condition-dependence on post-copulatory traits. In this chapter, I show that male testes and accessory gland size (after correcting for male body size) and sperm movement within the female reproductive tract are indeed condition-dependent, but such effects are determined early on in development and are irreversible at the adult stage. Therefore, this chapter demonstrates that male reproductive investment in *T. angusticollis* is likely constrained by holometabolous development, and suggests that programming of the cells that develop into the male germline occurs early on.

Next, Chapters Four and Five aimed to incorporate male investment in offspring quality through non-genetic factors into a condition-dependent life-history framework. While it has been accepted for many decades that males can influence the quality of their offspring through non-genetic factors like paternal care and nutrient-rich nuptial gifts and spermatophores (e.g., Gwynne & Simmons, 1990; Clutton-Brock, 1991; Badyaev & Hill, 2002), more recently it has been shown that males of some species can influence the quality of their offspring through more 'cryptic' non-genetic factors. Smaller amounts of proteins and peptides that are abundant in the seminal fluid (Chapman, 2001; Perry, Sirot, & Wigby, 2013), and epigenetic factors such as DNA methylation, chromatin structure and non-coding RNAs within the sperm and seminal fluid can have substantial effects on offspring quality (Grandjean *et al.*, 2015; Milekic

et al., 2015; Skinner, 2016). For example, a stressful environment can induce changes in the epigenome (e.g., DNA methylation patterns) which are then transmitted to the offspring and can have negative consequences for offspring health and fitness (reviewed in Danchin *et al.*, 2011; Soubry, 2015). In contrast, males from a good quality environment can produce better quality offspring (e.g., Bonduriansky and Head, 2007; Delcurto, Wu, & Satterfield, 2013; Evans *et al.*, 2017; Zajitschek *et al.*, 2017). Also, in some cases, fathers may be able to 'anticipate' offspring environment and produce offspring that are better suited to the anticipated environments (e.g., Crean, Dwyer, & Marshall, 2013; Jensen, Allen, & Marshall, 2014). However, it is not known if selection can favour investment in cryptic non-genetic factors that improve offspring quality, or if there are costs of building and maintaining such non-genetic factors. Understanding such costs is a fundamental question with broad implications for many areas of biology, including life-history theory. Therefore, in Chapter Four, I discuss the idea that in some systems, selection should favour male investment in the quality of his gametic epigenome, and examine the evidence that suggests that investment in epigenetic machinery could be costly. I then propose that if selection can favour male investment in the gametic epigenome, and if this investment is costly, we may expect such investment to be condition-dependent in the same way as other forms of paternal investment, whereby high condition individuals are more able to invest in epigenome quality.

In Chapter Five, I then take an idea prosed in Chapter Four that investment in offspring quality through non-genetic factors may trade-off with other costly traits and test if frequent mating results in a decline in offspring quality using the nerrid fly *Telostylinus angusticollis*. *T. angusticollis* males do not transfer a large and nutrient ejaculate, but previous studies have shown that high-condition males reared on a nutrient rich larval diet can produce larger offspring (Bonduriansky & Head, 2007), and this effect appears to be conferred through non-genetic factors within the seminal fluid rather than epigenetic factors tied directly to fertilisation (Crean, Kopps, & Bonduriansky, 2014). This chapter consists of two separate experiments where I test for a condition-dependent reduction in offspring quality (due to paternal larval diet) and male fecundity

after a bout of frequent mating, as well if mating rate decreases after a bout of frequent mating. In this chapter, I show that males appear to be altering their mating behaviour to avoid ejaculate depletion. I suggest that selection may have favoured equal ejaculate allocation across successive matings over a higher mating rate due to the high risk of sperm competition in this species. However, such a reproductive strategy prevented me from detecting a decline in male post-copulatory performance, including a decline in the ability to produce good quality offspring.

It must also be noted that I encountered a few problems during the second experiment of Chapter Five which prevented me from collecting some data that I had originally intended to collect. Initially, I had planned to collect fecundity and offspring quality data from each female that was mated to the focal males while testing for a reduction in mating rate with novel females. Unfortunately, the markings on the females disappeared during the reproductive performance assay which prevented me from differentiating the individual females. To overcome this problem, I pooled all the novel females together to examine total male fecundity and offspring quality across all the novel females that each focal male mated with. Additionally, for unknown reasons, only 3% of the eggs transferred to measure effects on offspring quality emerged into adults – allowing me to collect fecundity data but preventing any transgenerational measures. Overall, this chapter still demonstrates that males appear to be altering their reproductive behaviour after a bout of frequent mating, and that this may mitigate a depletion of the ejaculate components.

Finally, after examining the ejaculate depletion literature for Chapter Five and the data collected during the meta-analysis in Chapter Two, I was unable to find any studies that had examined if male condition affected the rate of sperm depletion. Furthermore, it remains unclear how nutrients and genotype influence males' ability to invest in sperm transfer across multiple matings. For example, Chapter Two clearly demonstrated that sperm quantity (i.e., the number of sperm produced/stored) is consistently condition-dependent (i.e., influenced by nutrient availability), suggesting that sperm depletion should also

be condition-dependent. Additionally, sexual traits, including post-copulatory traits, can have high additive genetic variance (e.g., Ward, 2000; Simmons & Kotiaho, 2002), and genetic effects can mediate responses to environment; thus, altering the strength of condition-dependent trait responses (Rowe & Houle, 1996; Hunt *et al.*, 2004). For Chapter Six, I was lucky enough to collaborate with Dr Stefan Lüpold at the University of Zürich on an experiment that tested for environmental effects of developmental nutrient availability and genetic (isoline) effects on sperm transfer across successive matings in *Drosophila melanogaster* that had been genetically engineered to express green florescent protein (Manier *et al.*, 2010). *Drosophila* species have exceptionally long sperm (Lüpold *et al.*, 2016), and sperm synthesis is expected to be costly, making males subject to sperm depletion (e.g., Linklater *et al.*, 2007). Additionally, the use of isolines with fluorescently labelled sperm enabled me to control for genetic background and count the number of sperm within the female reproductive tract – something that is difficult in many study systems. Surprisingly, in conjunction with dietary and genetic effects, I found evidence for an unknown source of variation that can have considerable effects on male reproductive performance, enabling some males to invest heavily in both mating rate and sperm transfer. I hypothesise that such effects could be the result of stochastic variation in gene expression or subtle differences in environment within replicate containers. This study demonstrates that sperm transfer is indeed condition-dependent, but there is more to male condition and ability to invest in reproduction than just diet and genes.

Overall, the studies in this thesis aim to provide a clearer understanding of male post-copulatory reproductive investment and condition-dependence. I demonstrate that biologically relevant sources of variation can alter how sperm and seminal fluid traits respond to nutrient limitation, and that it is important to consider such factors when drawing conclusions regarding post-copulatory condition-dependence. However, I also found evidence of an important additional source of variation in male post-copulatory investment in *Drosophila melanogaster*, suggesting that male ability to invest in reproduction is highly sensitive to factors other than diet and genes. Additionally, I posit that males from species that do not confer large and nutrient-rich ejaculates may also be

selected to invest in the quality of their offspring through more 'cryptic' non-genetic factors such as the quality of their epigenome or proteins and peptides in their seminal fluid, and that investment in these factors should be incorporated into a condition-dependent life-history framework. The combination of these five chapters provides new insights into male post-copulatory trait investment, particularly due to male condition, which will further our understanding of the evolution of different mating systems.

References

AMITIN, E.G. & PITNICK, S. (2007) Influence of developmental environment on male- and female-mediated sperm precedence in *Drosophila melanogaster*. *Journal of Evolutionary Biology* **20**, 381–391.

ANDERSSON, M. (1982) Sexual selection, natural selection, and quality advertisement. *Biological Journal of the Linnean Society* **17**, 375–393.

VAN LEEUWENHOEK, A. (1677) Observationes D. Anthonii Lewenhoeck, de natis'e semine genitali animalculis. *Philosophical Transactions of the Royal Society London* **12**, 1040–1046.

BADYAEV, A. V & HILL, G.E. (2002) Paternal care as a conditional strategy: distinct reproductive tactics associated with elaboration of plumage ornamentation in the house finch. *Behavioral Ecology* **13**, 591–597.

BONDURIANSKY, R. (2007) The evolution of condition-dependent sexual dimorphism. *American Naturalist* **169**, 9–19.

BONDURIANSKY, R. & HEAD, M. (2007) Maternal and paternal condition effects on offspring phenotype in *Telostylinus angusticollis* (Diptera: Neriidae). *Journal of Evolutionary Biology* **20**, 2379–2388.

CHAPMAN, T. (2001) Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity* **87**, 511–521.

CREAN, A.J., DWYER, J.M. & MARSHALL, D.J. (2013) Adaptive paternal effects? Experimental evidence that the paternal environment affects offspring performance. *Ecology* **94**, 2575–2582.

CREAN, A.J., KOPPS, A.M. & BONDURIANSKY, R. (2014) Revisiting telegony: offspring inherit an acquired characteristic of their mother's previous mate. *Ecology Letters* **17**, 1545–1552.

DANCHIN, E., CHARMANTIER, A., CHAMPAGNE, F.A., MESOUDI, A., PUJOL, B. & BLANCHET, S. (2011) Beyond DNA: integrating inclusive inheritance into an extended theory of evolution. *Nature Reviews Genetics* **12**, 475–486.

DARWIN, C. (1859) *On the origin of species by means of natural selection*. John Murry, London, UK.

DARWIN, C. (1871) *The Descent of Man and Selection in Relation to Sex*. John Murry, London, UK.

DEWSBURY, D.A. (1982) Ejaculate cost and male choice. *American Naturalist* **119**, 601–610.

DRONEY, D.C. (1998) The influence of the nutritional content of the adult male diet on testis mass, body condition and courtship vigour in a Hawaiian *Drosophila*. *Functional Ecology* **12**, 920–928.

EMLEN, D.J. (1997) Diet alters male horn allometry in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society B: Biological Sciences* **264**, 567–574.

EVANS, J.P., LYMBERY, R.A., WIID, K.S., RAHMAN, M. & GASPARINI, C. (2017) Sperm as moderators of environmentally induced paternal effects in a livebearing fish. *Biology Letters* **13**, 10–13.

FISHER, R.A. (1915) The evolution of sexual preference. *Eugenics Review* **7**, 115–123.

FISHER, R.A. (1930) *The genetical theory of natural selection*. Clarendon Press, Oxford, UK.

GODWIN, J.L., VASUDEVA, R., MICHALCZYK, L., MARTIN, O.Y., LUMLEY, A.J., CHAPMAN, T. & GAGE, M.J.G. (2017) Experimental evolution reveals that sperm competition intensity selects for longer, more costly sperm. *Evolution Letters* **1**, 102–113.

GRAFEN, A. (1990) Biological signals as handicaps. *Journal of Theoretical Biology* **144**, 517–546.

GRANDJEAN, V., FOURRE, S., DE ABREU, D.A.F., DERIEPPE, M.A., REMY, J.J. & RASSOULZADEGAN, M. (2015) RNA-mediated paternal heredity of diet-induced obesity and metabolic disorders. *Scientific Reports* **5**, 1–9.

GWYNNE, D.T. & SIMMONS, L.W. (1990) Experimental reversal of courtship roles in an insect. *Nature* **346**, 172–174.

HUNT, J., BUSSIÈRE, L.F., JENNIONS, M.D. & BROOKS, R. (2004) What is genetic quality? *Trends in Ecology and Evolution* **19**, 329–333.

JENSEN, N., ALLEN, R.M. & MARSHALL, D.J. (2014) Adaptive maternal and paternal effects : gamete plasticity in response to parental stress. *Functional Ecology*, **28**, 724–733.

JUDGE, K.A., TING, J.J. & GWYNNE, D.T. (2008) Condition dependence of male life span and calling effort in a field cricket. *Evolution* **62**, 868–878.

KAHRL, A.F. & COX, R.M. (2015) Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behavioral Ecology* **26**, 1502–1511.

KALDUN, B. & OTTI, O. (2016) Condition-dependent ejaculate production affects male mating behavior in the common bedbug *Cimex lectularius*. *Ecology and Evolution* **6**, 2548–2558.

KOTIAHO, J.S. (2000) Testing the assumptions of conditional handicap theory: costs and condition dependence of a sexually selected trait. *Behavioral Ecology and Sociobiology* **48**, 188–194.

LINKLATER, J.R., WERTHEIM, B., WIGBY, S. & CHAPMAN, T. (2007) Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. *Evolution* **61**, 2027–2034.

LÜPOLD, S., MANIER, M.K., PUNIAMOORTHY, N., SCHOFF, C., STARMER, W.T., LUEPOLD, S.H.B., BELOTE, J.M. & PITNICK, S. (2016) How sexual selection can drive the evolution of costly sperm ornamentation. *Nature* **533**, 535–538.

MANIER, M.K., BELOTE, J.M., BERBEN, K.S., NOVIKOV, D., STUART, W.T. & PITNICK, S. (2010) Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. *Science* **328**, 354–357.

MELO, M.C., ALMEIDA, F.R.C.L., CALDEIRA-BRANT, A.L., PARREIRA, G.G. & CHIARINI-GARCIA, H. (2014) Spermatogenesis recovery in protein-restricted rats subjected to a normal protein diet after weaning. *Reproduction, Fertility and Development* **26**, 787–796.

MILEKIC, M.H., XIN, Y., DONNELL, A.O., KUMAR, K.K., MALASPINA, D., MOORE, H., BRUNNER, D., GE, Y., EDWARDS, J. & PAUL, S. (2015) Age-related sperm DNA methylation changes are transmitted to offspring and associated with abnormal behavior and dysregulated gene expression. *Molecular Psychiatry* **20**, 995–1001.

MOLLER, A.P. & DELOPE, F. (1994) Differential costs of a secondary sexual character - an experimental test of the handicap principle. *Evolution* **48**, 1676–1683.

NUR, N. & HASSON, O. (1984) Phenotypic plasticity and the handicap principle. *Journal of Theoretical Biology* **110**, 275–297.

OLSSON, M.J., MADSEN, T. & SHINE, R. (1997) Is sperm really so cheap ? Costs of reproduction in male adders, *Vipera berus*. *Proceedings of the Royal Society B: Biological Sciences* **264**, 455–459.

PARKER, G.A. (1970) Sperm competition and its evolutionary consequences in

the insects. *Biological Reviews* **45**, 535–567.

PERRY, J.C., SIROT, L. & WIGBY, S. (2013) The seminal symphony: how to compose an ejaculate. *Trends in Ecology & Evolution* **28**, 414–422.

PERRY, J.C. & TSE, C.T. (2013) Extreme costs of mating for male two-spot ladybird beetles. *PLoS ONE* **8**, 1–5.

ROWE, L. & HOULE, D. (1996) The lek paradox and the capture of genetic variance by condition dependent traits. *Proceedings of the Royal Society B: Biological Sciences* **263**, 1415–1421.

ROWE, M., ALBRECHT, T., CRAMER, E.R.A., JOHNSEN, A., LASKEMOEN, T., WEIR, J.T. & LIFJELD, J.T. (2015) Postcopulatory sexual selection is associated with accelerated evolution of sperm morphology. *Evolution* **69**, 1044–1052.

SENTINELLA, A., CREAN, A. & BONDURIANSKY, R. (2013) Dietary protein mediates a trade-off between larval survival and the development of male secondary sexual traits. *Functional Ecology* **27**, 1134–1144.

SIMMONS, L.W. & KOTIAHO, J.S. (2002) Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution* **56**, 1622–1631.

SKINNER, M.K. (2016) Epigenetic transgenerational inheritance. *Nature Reviews* **12**, 68–70.

SOUBRY, A. (2015) Epigenetic inheritance and evolution: a paternal perspective on dietary influences. *Progress in Biophysics & Molecular Biology* **118**, 79–85.

CLUTTON-BROCK, T. H. (1991) *The Evolution of Parental Care*. Princeton University Press, Princeton, USA.

TRIVERS, R.L. (1972) Parental investment and sexual selection. B. Campbell (Ed.), *Sexual Selection and the Descent of Man*, 136–179. Aldine Publishing Company, Chicago, USA.

VERMEULEN, A., ENGELS, S. & SAUER, K.P. (2008) Maintenance of variance in sperm transfer rates in a scorpionfly: food availability, genetic basis, and heritability. *Behavioral Ecology and Sociobiology* **63**, 77–83.

WARD, P. (2000) Sperm length is heritable and sex-linked in the yellow dung fly (*Scathophaga stercoraria*). *Journal of Zoology* **251**, 349–353.

WIGBY, S., PERRY, J.C., KIM, Y.H. & SIROT, L.K. (2016) Developmental environment mediates male seminal protein investment in *Drosophila*

melanogaster. *Functional Ecology* **30**, 410–419.

ZAHAVI, A. (1975) Mate selection-a selection for a handicap. *Journal of Theoretical Biology* **53**, 205–214.

ZAJITSCHEK, F., ZAJITSCHEK, S. & MANIER, M. (2017) High-protein paternal diet confers an advantage to sons in sperm competition. *Biology Letters* **13**, 1–5.

Chapter Two

EFFECTS OF NUTRIENT LIMITATION ON SPERM AND SEMINAL FLUID: A SYSTEMATIC REVIEW AND META-ANALYSIS

Erin L Macartney, Angela J Crean, Shinichi Nakagawa, Russell Bonduriansky

Biological Reviews (2019). ***94****, 1722–1739*

ELM conceived and designed the project, collected and sorted the literature, extracted the data, analysed and wrote the manuscript. SN provided substantial guidance throughout data collection, extraction and analysis. RB provided valuable advice throughout, and AJC provided valuable advice during writing.

Abstract

Theory predicts that costly sexual traits should be reduced when individuals are in poor condition (i.e., traits should exhibit condition-dependent expression). It is therefore widely expected that male ejaculate traits, such as sperm and seminal fluid, will exhibit reduced quantity and quality when dietary nutrients are limited. However, reported patterns of ejaculate condition dependence are highly variable, and there has been no comprehensive synthesis of underlying sources of such variation in condition-dependent responses. In particular, it remains unclear whether all ejaculate traits are equally sensitive to nutrient intake, and whether such traits are particularly sensitive to certain dietary nutrients, respond more strongly to nutrients during specific life stages, or respond more strongly in some taxonomic groups. We systematically reviewed these potential sources of variation through a meta-analysis across 50 species of arthropods and vertebrates (from 71 papers and 348 effect sizes). We found that overall, ejaculate traits are moderately reduced when dietary nutrients are limited, but we also detected substantial variation in responses. Seminal fluid quantity was strongly and consistently condition dependent, while sperm quantity was moderately condition dependent. By contrast, aspects of sperm quality (particularly sperm viability and morphology) were less consistently reduced under nutrient limitation. Ejaculate traits tended to respond in a condition-dependent manner to a wide range of dietary manipulations, especially to caloric and protein restriction. Finally, while all major taxa for which sufficient data exist (i.e., arthropods, mammals, fish) showed condition dependence of ejaculate traits, we detected some taxonomic differences in the life stage that is most sensitive to nutrient limitation, and in the degree of condition dependence of specific ejaculate traits. Together, these biologically relevant factors accounted for nearly 20% of the total variance in ejaculate responses to nutrient limitation. Interestingly, body size showed considerably stronger condition-dependent responses compared to ejaculate traits, suggesting that ejaculate trait expression may be strongly canalised to protect important reproductive functions, or that the cost of producing an ejaculate is relatively low. Taken together, our findings show that condition-dependence of ejaculate traits is taxonomically widespread, but there are also many interesting, biologically relevant sources of variation that require further investigation. In

particular, further research is needed to understand the differences in selective pressures that result in differential patterns of ejaculate condition dependence across taxa and ejaculate traits.

Introduction

Male ejaculate traits are expected to exhibit condition-dependent expression

Persistent directional sexual selection can lead to trait exaggeration, such that morphological appendages, organs, physiological functions or behaviours whose expression is positively correlated with fitness evolve enlarged or elaborated expression (Darwin, 1859, 1871). However, expressing such exaggerated traits is thought to impose a variety of costs, including the costs of increased resource allocation and trade-offs with other organismal functions (Kotiaho, 2000). Consequently, theory predicts that trait exaggeration will also be associated with the evolution of heightened condition dependence, whereby high-condition individuals (i.e., those able to acquire more metabolic resources) are better able to invest in such costly traits (Andersson, 1982; Nur & Hasson, 1984; Rowe & Houle, 1996; Cotton, Fowler & Pomiankowski, 2004). Condition-dependence theory has been prominent in sexual selection theory for several decades, and heightened condition dependence has been demonstrated empirically in male secondary sexual traits such as ornaments and weapons (e.g., Moller & Delope, 1994; Emlen, 1997; Kotiaho, 2000). However, selection can also favour the exaggeration of sperm and semen traits (e.g., Cameron, Day & Rowe, 2007; Crudgington *et al.*, 2009; Rowe *et al.*, 2015; Lüpold *et al.*, 2016; Godwin *et al.*, 2017). Indeed, Lüpold *et al.* (2016) demonstrated that sperm traits can, in some cases, show even greater trait exaggeration than other secondary sexual traits. The expression of sperm and semen traits can impose non-trivial metabolic costs (e.g., Dewsbury, 1982; Olsson, Madsen & Shine, 1997; Marcotte, Delisle & McNeil, 2007; Perry & Tse, 2013; Lüpold *et al.*, 2016; Godwin *et al.*, 2017), resulting in the expectation that males in low condition should be less able to invest in sperm and semen traits compared to males in high condition. In other words, ejaculate traits may be expected to exhibit heightened condition dependence for the same reasons as other secondary sexual traits, such as ornaments and weaponry.

Parker (1970) recognised that sperm competition can generate sexual selection on sperm production, and proposed the 'raffle principle' whereby male fertilisation success is expected to depend on the number of sperm transferred

relative to competitors (Parker, 1990). Many subsequent theoretical and empirical studies have investigated selection on sperm number (i.e., the number of sperm in storage, or within an ejaculate) (Parker *et al.*, 1997; Gage & Morrow, 2003; Parker & Ball, 2005; Boschetto, Gasparini & Pilastro, 2011). Testes size, often used as a proxy for sperm production, has been shown to be correlated with levels of sperm competition (reviewed in Gage, 1994; Simmons & Fitzpatrick, 2012), and several studies have also shown that testes size (i.e., sperm production) and the number of sperm transferred can be correlated with paternity share (e.g., Engqvist *et al.*, 2007; Vellnow *et al.*, 2018); demonstrating that selection can indeed favour increased sperm number. However, in the last decade, there has been a shift of focus to selection on sperm quality (e.g., Gasparini *et al.*, 2010; Tourmente, Gomendio & Roldan, 2011; Gomendio *et al.*, 2006; Mehlis, Rick & Bakker, 2015), spurred on by Snook (2005). For example, increased sperm viability can be correlated with risk of sperm competition (e.g., Gomendio *et al.*, 2006), and increased sperm velocity can be associated with increased paternity (Boschetto *et al.*, 2011; Beausoleil *et al.*, 2012). Also, in some cases, increased sperm length has been correlated with sperm competition intensity (e.g., Gage, 1994; Radwan, 1996; LaMunyon & Samuel, 1999), but this effect is less consistent across taxa (reviewed in Simmons & Fitzpatrick, 2012) and is likely due to differences in selection environments among taxonomic groups (see Immler *et al.*, 2011; Liao *et al.*, 2018). For example, selection for sperm length is clearly favoured in *Drosophila* species but not in passerine birds and such differences are likely driven by differences in sperm competition mechanisms (i.e., raffle sperm competition in passerines but sperm displacement in *Drosophila*) (Immler *et al.*, 2011).

Non-sperm ejaculate components – i.e., the seminal fluid and seminal proteins and peptides –can also be highly important for male fitness and may be expected to evolve heightened condition dependence. Such components can increase sperm survival, as well as confer advantages in sperm competition by altering female physiology (Chapman, 2001; Ramm, Parker & Stockley, 2005; Avila *et al.*, 2011; South & Lewis, 2011; Crean, Adler & Bonduriansky, 2016). Additionally, components in the ejaculate can alter offspring development and quality (reviewed in Bromfield, 2014). Therefore, the non-sperm components

of the ejaculate are also likely to be under strong selection (Macartney, Crean & Bonduriansky, 2018*a*). A large body of literature has focused on males that confer large, nutrient-rich spermatophores and nuptial gifts (e.g., Gwynne, 1993; Jia, Jiang & Sakaluk, 2000; Perry & Rowe, 2010; Duplouy *et al.*, 2017). However, empirical evidence also suggests that selection can favour increased ejaculate expenditure in species where males do not transfer nutrient-laden ejaculates. For example, a study on *Drosophila pseudoobscura* demonstrated that increased risk of sperm competition selected for males with larger accessory glands (Crudgington *et al.*, 2009), and Linklater *et al.* (2007) demonstrated that *Drosophila melanogaster* males in male-biased populations invest more accessory gland products per mating compared to sperm, suggesting that intense sperm competition can also select for increased investment in the non-sperm ejaculate components (i.e., seminal fluid, and seminal proteins) (see also Cameron *et al.*, 2007).

Special challenges in research on ejaculate condition dependence

There is now much theoretical and empirical support for the hypothesis that sperm competition can select for increased investment in sperm and/or semen (e.g., Parker, 1990; Wedell, Gage & Parker, 2002; Gage & Morrow, 2003; Williams, Day & Cameron, 2005; South & Lewis, 2011), and that such investment can be strongly condition dependent (e.g., Tazzyman *et al.*, 2009). However, the nature and degree of sperm and semen condition dependence presents several complications and challenges for research. Ejaculate traits are typically cryptic: they encompass sperm quantity, morphology, motility and viability (i.e., quality), as well as seminal fluid quantity and composition. Variation in such traits can be subtle, and the energetic and resource-allocation costs of investment in such traits are difficult to quantify. Ejaculate investment is also highly multi-faceted. For example, sperm production comprises multiple different traits (i.e., number, morphology, motility and viability) that affect sperm quality and can be targets of selection (Lüpold & Pitnick, 2018), and seminal fluid contains hundreds of different proteins and peptides with varying (and largely unknown) functions (Avila *et al.*, 2011; Perry, Sirot & Wigby, 2013), and it is often difficult to identify the direct targets of sexual selection and predict which particular ejaculate traits should exhibit heightened

condition dependence (Poiani, 2006). The nature and degree of condition dependence of ejaculate traits therefore remain less well understood than the condition dependence of pre-copulatory secondary sexual traits such as ornaments and weapons.

A key source of variation in condition is nutrient availability, which determines the quantity of metabolic resources available for investment in all fitness-enhancing traits (Andersson, 1982; Rowe & Houle, 1996; Morehouse, 2014). However, like life-history traits such as ornaments and weaponry, ejaculate traits could exhibit complex patterns of plasticity. For example, ejaculate investment could be strongly dependent on specific nutrients, and particularly sensitive to nutrient limitation at specific ontogenetic life stages. Ejaculate investment strategies are also likely to vary among (and sometimes within) species. Such factors could contribute to variable results of empirical studies. While many studies have indeed demonstrated condition-dependent responses of male sperm and semen traits through experiments manipulating nutrient availability (e.g., Droney, 1998; Rahman, Kelley & Evans, 2013; Vega-Trejo, Jennions & Head, 2016; Dávila & Aron, 2017), some studies have found that males may increase their investment in ejaculate traits when nutrients are reduced (e.g., Simmons, Tomkins & Hunt, 1999; Perry & Rowe, 2010; Mehlis *et al.*, 2015), while others have found no effect of diet on such traits (e.g., Sitzmann *et al.*, 2010; Sullivan, Brown & Clotfelter, 2014). Identifying and accounting for key sources of biological variation in ejaculate investment could help to make sense of such variable results.

Different sperm and seminal fluid traits could be subject to varying selection pressures within and among species, resulting in different levels of costliness and thus differences in condition dependence. For example, there is generally thought to be a trade-off between sperm quality and quantity, and taxonomic differences in the selection environment can alter this trade-off (Immler *et al.*, 2011; Liao *et al.*, 2018). Within species, Bunning *et al.* (2015) demonstrated that sperm number and sperm viability increased linearly with an increase in protein and carbohydrates in the cockroach *Nauphoeta cinerea* and investment peaked at the same protein:carbohydrate ratio, but sperm number responded more

strongly to nutrient availability than did sperm viability. Additionally, males in high *versus* low condition may employ differing reproductive strategies, and therefore exhibit differential responses to nutrient limitation. For example, Perry & Rowe (2010) found that male ladybirds (*Adalia bipunctata*) alter their investment in sperm and non-sperm components depending on their condition, with high-condition males (i.e., males reared on a nutrient-rich diet) investing more in non-sperm components of the ejaculate, and low-condition males (i.e., males reared on a nutrient-poor diet) investing more in absolute sperm number. This suggests that different ejaculate traits are differentially sensitive to nutrient abundance, and that males may employ different investment strategies based on their condition and mating system. Hence, the strength and direction of a trait's response to diet can vary depending on the species and on the particular ejaculate trait measured.

Sources of variation in ejaculate condition dependence

Most studies examining the condition dependence of male ejaculate traits have manipulated dietary intake of nutrients. Many such studies have manipulated total calories without altering nutrient ratios (e.g., Vermeulen, Engels & Sauer, 2008; Kahrl & Cox, 2015; Mehlis *et al.*, 2015; Kaldun & Otti, 2016; Vega-Trejo *et al.*, 2016). However, particular macro- or micronutrients may be particularly important for ejaculate trait expression. For example, protein is essential for oogenesis (Chippindale & Leroi, 1993; Adler *et al.*, 2013), but there is also evidence to suggest that protein can be important for male sperm and semen traits (e.g., Droney, 1998; Melo *et al.*, 2014; Dávila & Aron, 2017). Other studies have suggested that micronutrients such as carotenoids, amino acids and vitamins can affect ejaculate trait expression (Lederhouse *et al.*, 1990; Locatello *et al.*, 2006; Lambrot *et al.*, 2013; Yossa *et al.*, 2015; Tomášek *et al.*, 2017). But we do not know how different dietary components contribute to the variation in ejaculate responses.

The ontogenetic stage when nutrients are limited (i.e., during juvenile development *versus* during the adult stage) could also be very important. The adult diet could affect ejaculate trait expression because spermatogenesis typically occurs throughout adulthood, and the amount of metabolic resources

available to adult males has been shown to influence investment in ejaculate traits in some species (e.g., Droney, 1998; Perry & Rowe, 2010; Evans, Rahman & Gasparini, 2015; Kahrl & Cox, 2015; Bailey, Legan & Demas, 2017). This may be particularly true in animals that have indeterminate growth, such as fish, molluscs and some reptiles. Such species require energy throughout life to maintain growth and reproduction, and energy gained through diet may be reallocated to growth instead of reproduction when nutrients are scarce (Heino & Kaitala, 1999). But the developmental environment can alter metabolic pathways in adults (e.g., Gheorghe *et al.*, 2010), and differences in developmental nutrients could change how resources are mobilised and allocated to sperm and semen traits. Investment in adult reproductive traits may be programmed during development (e.g., *via* changes in epigenetic factors such as DNA methylation, chromatin structure and non-coding RNAs), and this may depend on nutrient availability during development (Macartney *et al.*, 2018*a*). Also, effects of nutrient limitation during development may be more prevalent in some taxa. For example, holometabolous insects rely on developmental nutrient acquisition for investment in adult reproductive traits (Boggs, 1981), including ejaculate traits (e.g., Dávila & Aron, 2017; Macartney *et al.*, 2018*b*). However, there has been no systematic assessment of how male ejaculate traits respond to nutrient limitation at different ontogenetic stages, or how these effects differ among taxa.

Systematically reviewing ejaculate condition dependence

Although many experimental studies have investigated the effects of nutrition on expression of a range of ejaculate traits in a diverse range of species, there has, as yet, been no systematic synthesis of these data. We conducted a systematic review with comparative meta-analyses by combining published data across a variety of species where male condition was manipulated by experimentally limiting dietary nutrient availability relative to a 'standard' or '*ad libitum*' baseline. We then used meta-regression and sub-analyses to determine: (*i*) which male ejaculate traits respond most strongly to nutrient limitation, (*ii*) which nutrients have the strongest effects on ejaculate trait expression, (*iii*) whether the degree of nutrient limitation alters the strength of condition-

dependence, (*iv*) what ontogenetic life stage (i.e., juveniles or adults) is most sensitive to nutrient limitation, and (*v*) how these effects vary across taxa.

Studies of condition dependence of morphological traits such as signals and weapons typically compare the responses of these traits with the response of body size (Cotton *et al.*, 2004). While we do not have *a priori* predictions of how most ejaculate traits should scale with body size, some ejaculate traits (such as testes size and therefore sperm quantity) are usually correlated with body size (e.g., Locatello *et al.*, 2008; O'Dea, Jennions & Head, 2014; Macartney *et al.*, 2018*b*; but see Mautz, Møller & Jennions, 2013). We therefore conducted parallel analyses of treatment effects on ejaculate traits and on body size, allowing us to compare the degree of condition dependence in ejaculate traits with the degree of condition dependence in overall growth.

Methods

Data collection and effect-size extraction

ISI Web of Science and *Scopus* were used to search for studies between January 1900 and June 2017 that manipulated diet with an *ad libitum* or control diet and a diet-limitation manipulation and measured at least one ejaculate trait. Topics (i.e., title, key words and abstracts) were searched using the search string (condition OR diet* OR nutrient* OR food OR resource*) AND (sperm* OR semen OR ejaculate OR test?s). This resulted in 1086 papers from *ISI Web of Science* and 1493 papers from *Scopus*. 2117 papers remained after removing duplicates (this number is inflated as some replicates were missed due to differences in title formatting between databases). Titles and abstracts were then screened using Abstrackr (Wallace *et al.*, 2012) which uses machine learning to help order papers from most to least relevant. Of the 2117 papers screened, 138 papers met our initial selection criteria where diet was experimentally manipulated and at least one ejaculate trait was measured. All studies included in this meta-analysis were laboratory studies on animals reared in the laboratory. We excluded all studies on humans as it is not possible to manipulate human nutrient intake or to control for other environmental variables to the same extent as in laboratory studies. We also excluded all experiments on domestic and agricultural animals due to the likely high

artificial selection for reproductive output that may result in inflated condition dependence that is not representative of ejaculate traits in natural populations. We also excluded diets where increased consumption likely results in decreased condition rather than an increase in condition such as males fed 'Western diets' (i.e., high trans- and saturated-fats, and refined sugar), toxins and carcinogens. We identified another 26 papers through backward and forward searching (i.e., systematically checking the bibliography of the 138 studies that met our initial search criteria and checking for relevant papers that had cited those 138 studies). This resulted in 164 papers for further screening. Briefly, we excluded all ejaculate traits that could be the result of female differential allocation or cryptic female choice rather than male investment in the trait, such as paternity share and offspring quality, as well as studies that reported gonado-somatic indices or traits corrected for body size as these may confound effects of diet on body size with effects on ejaculate traits. We also excluded studies that had ambiguous diet manipulations where we were unable to tell which diet would be an *ad libitum*/control or a limited diet, and studies with missing data (for three out of six studies with missing data, we were unable to obtain the data after contacting the authors). This resulted in 71 studies with 348 ejaculate trait effect sizes across 50 species [see Fig. 1 for the preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram, Fig. 2. for the distribution of taxa across the studies, and online Supporting information. Fig. S1 for a phylogenetic tree of the included species).

Effect-size calculation

Standardised mean differences (SMDs) were used to compare the 'high' and 'low' diets and were calculated as Cohen's *d* coefficients:

$$SMD = \frac{mean_c - mean_l}{s_{pooled}},$$

$$s_{pooled} = \sqrt{\frac{(n_c-1)s_c^2+(n_l-1)s_l^2}{n_c+n_l-2}}.$$

Where $mean_c$ are the mean measures of a trait for individuals from the *ad libitum*/control diet treatment, $mean_l$ are the mean measures of a trait for

individuals from the nutrient-limited diet treatment, and S_{pooled} are the pooled standard deviations whereby more weight is given to groups of individuals with a larger sample size (n), and s_c^2 and s_l^2 are the standard deviations of the trait in individuals from the control and limited diet treatments, respectively. An effect size (SMD) was calculated for each trait across all papers that remained in the meta-analysis. We used conventional benchmarks for what is considered a 'high' (0.8/–0.8), 'moderate' (0.5/–0.5) or 'low' (0.2/–0.2) effect of diet on traits (Cohen, 1988).

Sampling variance for each SMD was calculated as:

$$S_{d=}^2 \frac{n_c + n_l}{n_c n_l} + \frac{SMD^2}{2(n_{c+} n_l)}.$$

Descriptive statistics such as means and standard deviations/errors were used to calculate SDM where possible (see Noble *et al.*, 2017). If descriptive statistics were not available, inferential statistics such as *F* and χ^2 values were used if the assumption of non-independence was met (Noble *et al.*, 2017). If experiments included a split-brood design and therefore resulted in non-independence due to genetic relatedness of individuals within a treatment, we used family number instead of individual number as n when calculating S_{pooled} in order to calculate a more conservative estimate of the effect of diet on traits.

Moderator variables for meta-regression

Moderator (predictor/explanatory) variables were included in meta-regression analyses to test for correlations between moderator variables and ejaculate trait expression when nutrients are limited. The moderator variables included in the meta-regression analyses were: the degree of diet limitation (%), the type of ejaculate trait (e.g., sperm and seminal fluid quantity, and measures of sperm quality), the ontogenetic stage at which diet was manipulated (i.e., juveniles *versus* adults), and the type of diet manipulation (e.g., reduction of specific nutrients, or overall food reduction). The degree of diet reduction was calculated as a per cent of food limitation relative to the fully fed/control diet for the studies that provided quantitative details in their methods (mean ± S.D.

% reduction = 56.89 ± 31.76%, range = 0.3–100%). This was then z_r-transformed to improve interpretability (*sensu* Schielzeth, 2010). Ejaculate traits were divided into six categories: total sperm length (note that we did not include effects on multiple parts of the sperm such as flagella, midpiece and head length as these are expected to be highly correlated and the number of studies that measured these individual parts was too low for analysis), sperm movement (including sperm velocity, flagella beat frequency and the per cent that were motile), sperm quantity (including total number or concentration of stored sperm or sperm in an ejaculate) (note that there were not enough data to separate sperm number traits into sperm stored within the male *versus* sperm transferred to the female within an ejaculate so these were pooled), sperm normality (if the sperm were viable and/or of normal morphology), seminal fluid quantity (including non-sperm ejaculate size and accessory gland size), and traits that encompass multiple ejaculate components that were not separated into individual components such as sperm and semen quantity (i.e., testes size and spermatophore size). The ontogenetic life stage when nutrients were limited was divided into two categories: before sexual maturity (juveniles) or after sexual maturity (adults). Diet manipulations were divided into six categories: total quantity of food (including manipulations of concentration), protein, carbohydrates, fats [polyunsaturated fatty acids (PUFAs) and monounstaturated fatty acids (MUFAs)], micronutrients (i.e., dietary carotenoids, amino acids, vitamins and minerals), and food quality (i.e., subjective manipulations of diet quality specific to the study species of interest). We excluded the effects of carbohydrates in all analyses except the sub-analyses on mammals as carbohydrate manipulations were only carried out in mammal studies. Where effects of diet manipulation on body size were reported along with effects on ejaculate traits, we used these body-size results to carry out parallel analyses of the condition dependence of body size for comparison with responses of ejaculate traits.

Moderator-variable levels required a sample size of >5 (k) observations to be included in analyses. Any moderator variables (i.e., a specific diet manipulation or trait) that were only investigated in one taxon were removed from the overall analysis to avoid confounding effects of other moderator variables with

effects of taxon, but these moderator variables were included in the taxonomic sub-analyses (Appendix S1). Sub-analyses of the overall effect of diet on ejaculate traits and meta-regression of moderator variables were also completed on arthropods, mammals and fish in order to assess if any effects differ among taxa. Sub-analyses were not carried out on other taxa (e.g., birds and reptiles) as studies on these taxa were too few for meaningful conclusions (see Fig. 2).

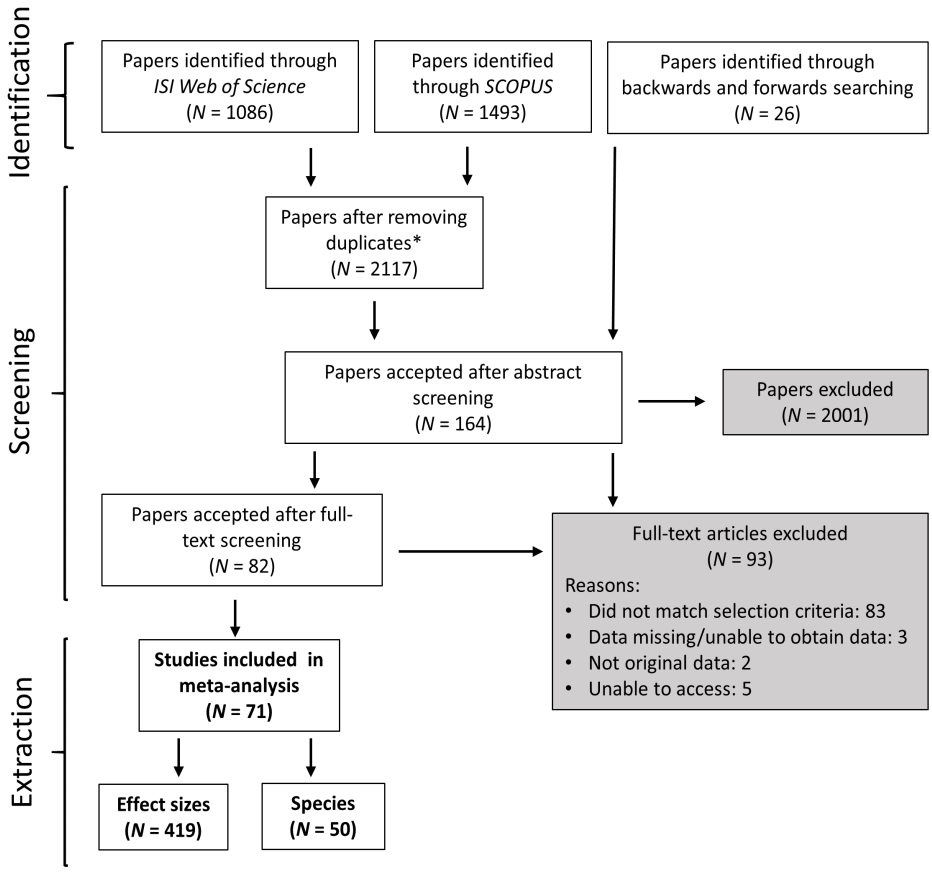


Figure 1. PRISMA flow diagram of the data-collection process. The number of papers remaining after each stage of the selection process is shown in each identification box, as well as the number of papers excluded and the reasons for exclusion for the papers that remained until final screening. Effect-size number is after processing data (i.e., collapsing repeated measures). *Some duplicates were missed due to differences in title formatting between databases.

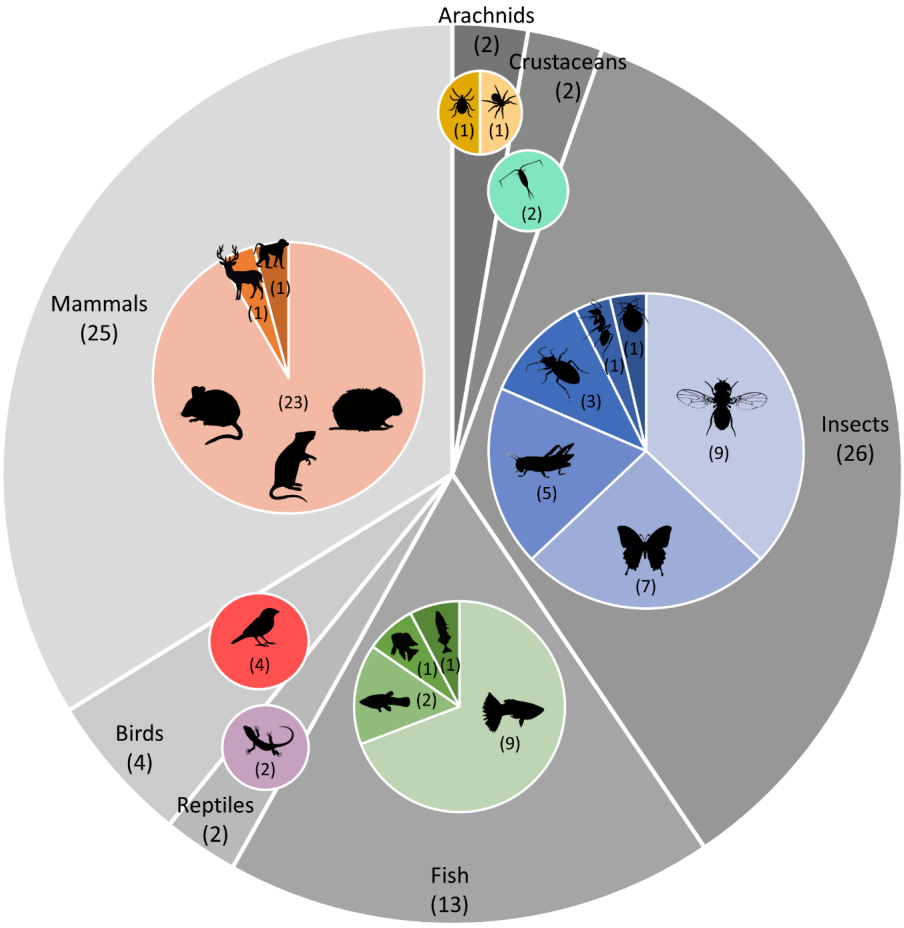


Figure 2. Pie chart showing the number of studies included in this meta-analysis within each taxonomic group. The outer pie chart depicts the number of studies within large taxonomic groups used in this analysis: Arachnids, Crustaceans, Insects, Fish, Reptiles, Birds, and Mammals (note that Insects, Arachnids and Crustaceans were analysed together as Arthropods in the sub-analysis; results remain unchanged if these groups are analysed separately). The inner pie charts depict the number of studies conducted within each order. Moving clockwise from light to dark: Arachnids: Araneae, Trombiformes; Crustaceans: Copepoda; Insects: Diptera, Lepidoptera, Orthoptera, Coleoptera, Hymenoptera, Hemiptera; Fish: Cyprinodonteformes, Cyprinoformes, Cichliformes, Gasterosteiformes; Reptiles: Squamata; Birds: Passeriformes; Mammals: Rodentia, Artiodactyla, Primates.

Statistical analyses

Meta-analysis and meta-regression

Meta-analyses and meta-regressions were carried out using multi-level, mixed-effects models in R version 1.1.447 using package metafor (Viechtbauer, 2010) as these models are generally required for ecological and multispecies meta-analyses (Nakagawa *et al.*, 2017). Initially, we ran models without any moderator variables to test for the overall mean effect of nutrient limitation on ejaculate trait expression and body size (separate models). We controlled for ejaculate trait co-linearity within study individuals (i.e., it would be expected that different ejaculate traits measured from the same study individuals would be correlated to some degree) by including a variance–covariance matrix for multiple traits measured from the same individuals in the model (see Nakagawa *et al.*, 2017; Noble *et al.*, 2017). We also originally included a variance–covariance matrix for phylogenetic relatedness of the included species as a random effect in the model (Chamberlain *et al.*, 2012), but this was later removed as its inclusion did not improve model fit and the phylogenetic signal was very weak (see Section III). Effects of nutrient restriction remained robust (Appendix S2). Animal group ID (i.e., the unique identifier for groups of animals within one set of SMD calculations), species (i.e., to control for differences between species, but not accounting for phylogenetic relatedness) and effect size ID (i.e., an observation-level unique identifier for each SMD calculated) were included as random-effects. I^2 was calculated as a measure of total heterogeneity between SMDs that is not attributed to sampling error (Higgins *et al.*, 2003). We expected high heterogeneity (I^2 = 60–90%) as the current literature on the effects of diet on ejaculate traits suggest that effects vary considerably among studies, and high levels of heterogeneity are generally expected in ecological studies (Higgins *et al.*, 2003; Senior *et al.*, 2016).

Next, we added single moderator variables (see Section II.3) to the models to test for their effects in mediating the response of traits to nutrient limitation. We quantified how much variance in responses to nutrient limitation was explained by each individual moderator by calculating its marginal R^2, *sensu* Nakagawa & Schielzeth, 2013. We then tested a full model containing all

moderator variables to calculate how much variance in responses to nutrient limitation is explained by all the moderator variables together.

In addition to the analysis of nutrient limitation effects on male body size (based on all effect sizes for body size from all papers included in this meta-analysis), we used studies that measured both ejaculate traits and body size to conduct a pair-wise comparison between effects of nutrient limitation on ejaculate traits *versus* body size. This model included animal group ID, species and effect-size ID as random effects, as well as the variance–covariance matrix to control for trait co-linearity within study individuals.

Pairwise comparisons were conducted using the package multcomp (Hothorn, Bretz & Westfall, 2008) and the function glht.

Taxonomic sub-analyses

Finally, we completed sub-analyses on the taxonomic groups for which enough data were available from multiple studies (arthropods, mammals, and fish), to determine if ejaculate responses to nutrient limitation differed between these taxonomic groups. Separate sub-analyses were also completed on insects and rodents, but results were qualitatively similar, so taxonomic groups were expanded to 'mammals' (including studies on rodents, cervids and primates) and 'arthropods' (including studies on insects, crustaceans and spiders). Sperm movement was excluded from the arthropod sub-analysis, sperm size was excluded from the mammal sub-analysis, and testes size and seminal fluid quantity were excluded from the fish sub-analysis due to small sample sizes ($N \leq 5$). All diet manipulations except 'quantity' and 'micronutrient' (carotenoid) manipulations were excluded from the fish sub-analysis because few studies examined other types of dietary effects. All results are presented as SMD (Cohen's *d*) and 95% credible intervals (CI).

Publication bias and sensitivity analysis

The R package MCMCglmm (Hadfield, 2010) was used for publication bias analyses. Publication bias was assessed using multiple methods. First, we assessed funnel asymmetry of the 'meta-analytic' residuals (*sensu* Nakagawa &

Santos, 2012), calculated from the full model and plotted against 'precision' (i.e., the inverse of standard error). Note that the use of the meta-analytic residuals fulfils the independence assumption. Also, these residuals are less affected by heterogeneity; the funnel asymmetry can be due to true heterogeneity in data (i.e., unexplained variation). We then used Egger's regression tests for deviations in funnel asymmetry (i.e., using linear regression to test for a significant deviation of the *y*-intercept from 0) (Egger *et al.*, 2015), and a 'trim-and-fill' test to predict 'missing' (i.e. unpublished) studies from the literature based on funnel asymmetry (Duval & Tweedie, 2000). Next, we tested for a correlation of publication year with the size of the SMDs by including publication year as a single moderator variable in the model.

These assessments of publication bias on ejaculate traits were conducted with one low-precision (i.e., small sample size) study on the house mouse (*Mus musculus*) (Chinoy, Mehta & Jhala, 2006) removed from the analyses, as they reported very large effects of nutrient limitation on ejaculate traits and this resulted in issues with running the Egger's regression tests and trim-and-fill test on the meta-analytic residuals as suggested by Nakagawa & Santos (2012) as it skewed the mean meta-analytic residuals away from zero. However, this study had low weight in the meta-analysis, meta-regression and sub-analyses on mammals due to its small sample size; there was no substantial difference in the other reported results with this study removed (see Appendix S3 for meta-analysis, meta-regression and mammal sub-analyses with this study removed). Additional sensitivity analyses were conducted where one animal group at a time was taken out of the data set and a new SMD and 95% CI were calculated for the global meta-analytic model. Assessments of publication bias and 'leave-one-group-out' sensitivity analyses were conducted separately for ejaculate traits and body size (Appendix S4).

Results

Does nutrient limitation cause an overall reduction in male ejaculate traits?

On average, across all ejaculate traits, taxa, diet manipulations, and ontogenetic life stages, a reduction in nutrient intake resulted in a significant and 'moderate' decrease in male ejaculate traits (SMD [non-phylo model] = –0.525; CI = –

0.684, –0.338) (Fig. 3). Including phylogenetic relatedness as a random effect did not improve model fit according to the change (Δ) in Akaike Information Criterion (ΔAIC = 1.210). There was no evidence of a phylogenetic signal (Pagel's Lambda = < 0.001%), and effects of nutrient restriction on ejaculate traits remained moderate and robust with phylogeny omitted from the model (SMD [phylo model] = –0.496; CI = –0.854, –0.138) (see also Appendix S2). However, as expected, there was a high amount of heterogeneity in ejaculate responses to a reduced diet (I^2[total] = 89.6%), with effect-size ID (i.e., observation-level SMD) accounting for 70.0% of the variance, different experimental animal groups (i.e., groups of animals from different experiments) accounting for 14.7% of the variance, and differences between species that were not accounted for by phylogenetic relatedness accounting for 5.0% of the variance.

The high amount of heterogeneity in male ejaculate traits to nutrient limitation suggests that other factors modulate the responses of such traits to a reduction in nutrient intake. Therefore, we attempted to explain this variation using meta-regression analyses of several moderator variables: the degree of nutrient reduction, the life stage at which nutrients were reduced, the type of nutrient manipulation, and the type of ejaculate trait measured. Overall, these moderator variables explained 19.0% (R^2) of the variance in ejaculate trait responses to nutrient limitation (see Appendix S4 for overall effect and meta-regression on male body size).

Are ejaculate traits sensitive to the degree of nutrient limitation?

We only detected a small and non-significant effect of the degree of nutrient limitation on ejaculate traits (SMD [degree nutrient limitation] = –0.015; CI = –0.227, 0.198, R^2 = 0.6%, k = 253). However, this may be due to the limited range of reported diet reductions. Of the studies that reported the exact reduction in diets, 67.90% reported a diet reduction of greater than 50% compared to the control diet.

Are certain ejaculate traits more sensitive to nutrient limitation?

Overall, seminal fluid quantity was reduced to a large extent when nutrients were limited.

Spermatophore/testes size and sperm quantity were moderately reduced when nutrients were limited. Sperm movement and sperm length had a small, negative response to nutrient limitation, and sperm normality was largely unaffected (Fig. 3). The type of ejaculate trait accounted for 8.3% of the variance in responses (R^2).

Are ejaculate traits more sensitive to nutrient limitation at the juvenile or adult stage?

Nutrient limitation at both juvenile and adult life stages resulted in a moderate, significant decrease in ejaculate traits (R^2 = 0.8%; Fig. 3). The mean effect of nutrient limitation in juveniles was slightly greater than the effect of nutrient limitation in adults, but this small difference was not significant (SMD [adult–juvenile comparison] = –0.178; CI = –0.492, 0.167).

Are male ejaculate traits sensitive to specific dietary components?

A reduction in total food quantity and dietary protein resulted in a moderate, significant decrease in ejaculate trait expression. A reduction in PUFA and MUFA fats, micronutrients (e.g., dietary carotenoids, vitamins, minerals and amino acids), and diet quality (i.e., reductions in diet quality specific to the study species) resulted in small, non-significant reductions in ejaculate trait expression (Fig. 3). The type of nutrient limitation accounted for 4.4% of the variance in responses (R^2).

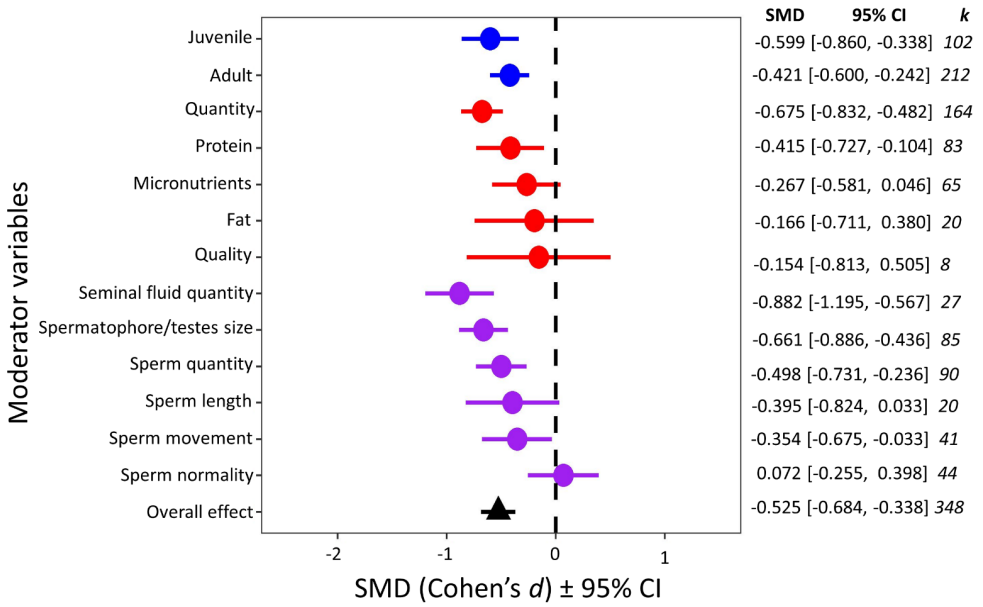


Figure 3. Forest plot displaying the overall effect (black triangle) of nutrient limitation on male ejaculate traits expression, as well as the effects of ontogenetic life stage (blue), type of diet manipulation (red), and the effects on different ejaculate traits (purple). Text on the right displays the standardised mean difference (SMD, Cohen's *d*), 95% credible interval (CI), and the number of observations for each moderator variable (*k*).

Are these results consistent across taxa?

Ejaculate trait expression was moderately and significantly reduced with nutrient limitation in arthropods (SMD [$arthropod_{Total}$] = –0.442; CI = –0.662, –0.222) and strongly reduced in mammals (SMD [$mammal_{Total}$] = –0.859; CI = –1.155, –0.563). There was also a small and near-significant reduction in fish (SMD [$fish_{Total}$] = –0.254, CI = –0.577, 0.067) (Fig. 4). Therefore, the strength of ejaculate condition dependence may be taxon specific, but the difference in effects may also reflect differences in sample size or be due to underlying differences in experimental design used within different taxa. Most ejaculate traits across taxa were reduced when diet was reduced (Fig. 4). However, our analysis also revealed some interesting exceptions. In particular, sperm and seminal fluid quantity were significantly reduced in arthropods and mammals but sperm quantity was not significantly reduced in fish (no studies measured seminal fluid quantity in fish). Sperm length appeared to be largely unaffected in arthropods but was significantly reduced in fish (few studies measured

sperm length in mammals), and sperm normality was unaffected in both arthropods and mammals but was significantly reduced in fish (Fig. 4). Therefore, the condition-dependent trait responses of fish sperm quality and quantity were largely opposite to those of arthropods and mammals.

Most diet manipulations resulted in at least a slight decrease in ejaculate trait expression across arthropods, mammals, and fish. A reduction in the total quantity of food resulted in a consistently significant, moderate to large decrease in ejaculate traits across all taxa, and a reduction in protein resulted in a significant, moderate to large decrease in ejaculate traits in arthropods and mammals (protein was not included in the fish sub-analysis due to a lack of data). We also detected a large, significant negative effect of carbohydrates in mammals (carbohydrates were not manipulated in arthropod or fish studies), albeit with large variation. Diet quality (i.e., qualitative reductions in diet specific to the diet of the study species) did not result in a substantial decrease in ejaculate traits (specific to arthropod studies), and micronutrients such as carotenoids (specific to the fish studies), amino acids, vitamins and minerals do not appear to be important for arthropod or fish sperm and semen traits but resulted in a moderate, marginally non-significant reduction in ejaculate traits in mammals (Fig. 4).

Nutrient limitation at as juveniles resulted in a significant decrease in ejaculate trait expression in both arthropods and mammals, and nutrient limitation as adults resulted in a relatively weaker, significant reduction in ejaculate traits in arthropods and a marginally non-significant reduction in mammals. The effect of juvenile nutrient limitation was significantly larger than the effect of adult nutrient limitation in mammals (SMD [mammal adult–juvenile comparison] = –0.962; CI = –1.845, –0.109), but the difference between adult and juvenile nutrient limitation was non-significant in arthropods (SMD [arthropod adult–juvenile comparison] = –0.311; CI = –0.786, 0.163). By contrast, in fish, nutrient limitation as adults resulted in a significant reduction in ejaculate traits but nutrient limitation as juveniles largely unaffected ejaculate traits, however this difference was not significant (SMD [fish adult-juvenile comparison] =

0.448; CI = –0.0410, 0.937) (Fig. 4; see Appendix S4 for taxonomic sub-analyses on male body size).

Effect of nutrient limitation on male body size

We carried out a parallel meta-analysis on nutrient effects on body size, using effects reported in the same studies that we included in our meta-analysis of effects on ejaculate traits. We found that a decrease in nutrient intake resulted in a large reduction in body size (SMD [total] = –1.359; CI = –1.779, –0.940, k = 71, also see Appendix S4).

The estimated effect of nutrient limitation on body size substantially exceeded the estimated effect on ejaculate traits. To verify this difference, we analysed the subset of studies that reported effects of nutrient limitation on both ejaculate traits and body size within the same study. This pair-wise comparison showed that the difference in trait responses between ejaculate traits and body size was indeed significant (SMD [ejaculate–body size comparison] = –0.708; CI = –0.955, –0.462).

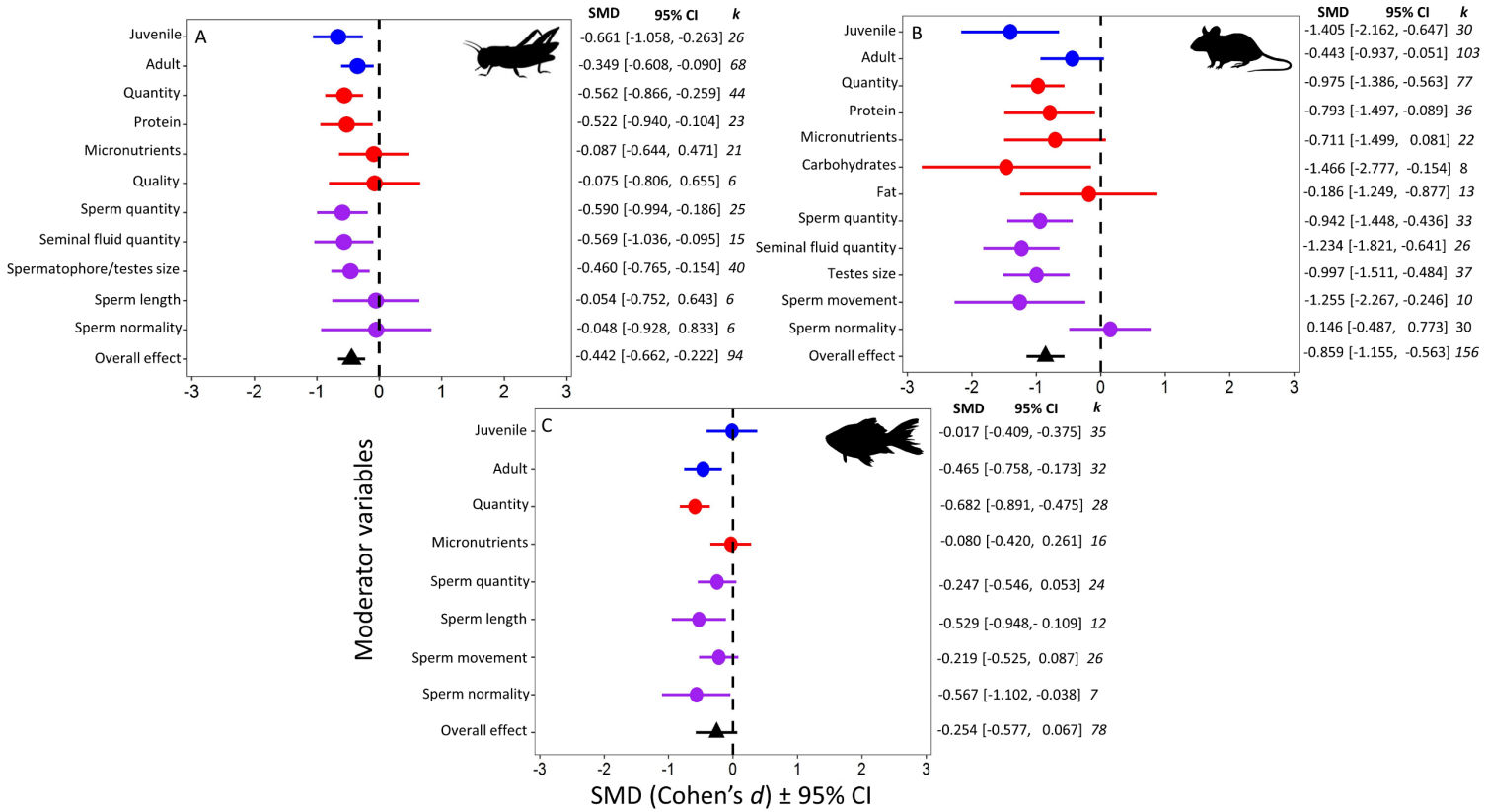


Figure 4. Forest plots displaying the overall effect (black triangles) of nutrient limitation on male ejaculate traits expression across taxa (A, arthropods; B, mammals; C, fish), as well as the effects of ontogenetic life stage (blue), type of diet manipulation (red), and the effects on different ejaculate traits (purple). Text on the right displays the standardised mean difference (SMD, Cohen's *d*), 95% credible interval (CI), and the number of observations for each moderator variable (*k*).

Publication bias and sensitivity analysis

Statistical assessment of our ejaculate trait data, after controlling for heterogeneity using 'meta-analytic' residuals, suggests that there is some publication bias in the literature; this was confirmed with the Egger's regression test for funnel asymmetry (β [intercept] = –0.621, S.E. = 0.248, t = 2.730 = –2.509, P = 0.013), and the 'trim-and-fill' method added three points to the right side (positive effects) of the funnel plot (Fig. 5). Funnel asymmetry suggests that several low-precision studies (i.e., studies with small sample sizes) that report a strong increase in male ejaculate traits when fed a nutrient-reduced diet are missing from the published literature. There was also a significant effect of publication year on the SMDs of male ejaculate traits where the size of the SMD became smaller with publication year (β [publication year] = –0.0003, CI = –0.0003, –0.0002) (Fig. 6), and publication year explained 5.76% (R^2) of the variance in effect sizes (see Appendix S4 for publication bias analyses on body size).

'Leave one group out' sensitivity analyses showed that effects of nutrient limitation on ejaculate traits (Fig. S6) and body size (Fig. S7) remain robust when any one group of study animals is omitted from the meta-analytic data set [also see Appendix S3 for analyses with the large outlier Chinoy *et al.* (2006) removed].

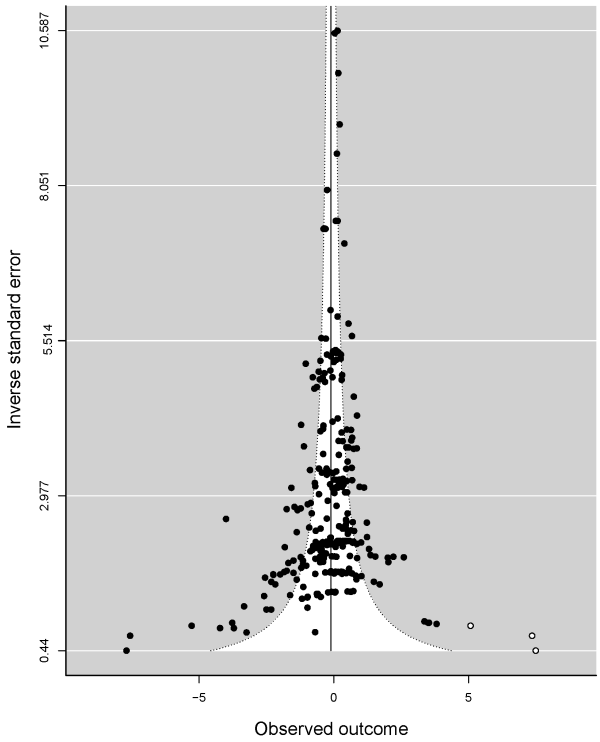


Figure 5. Funnel plot to test for publication bias of nutrient limitation on male ejaculate traits. The *x*-axis indicates the meta-analytic residuals, and the *y*-axis indicates the inverse standard error (precision). The black dots represent published data, and the white dots represent 'missing' data as calculated by the trim-and-fill analysis.

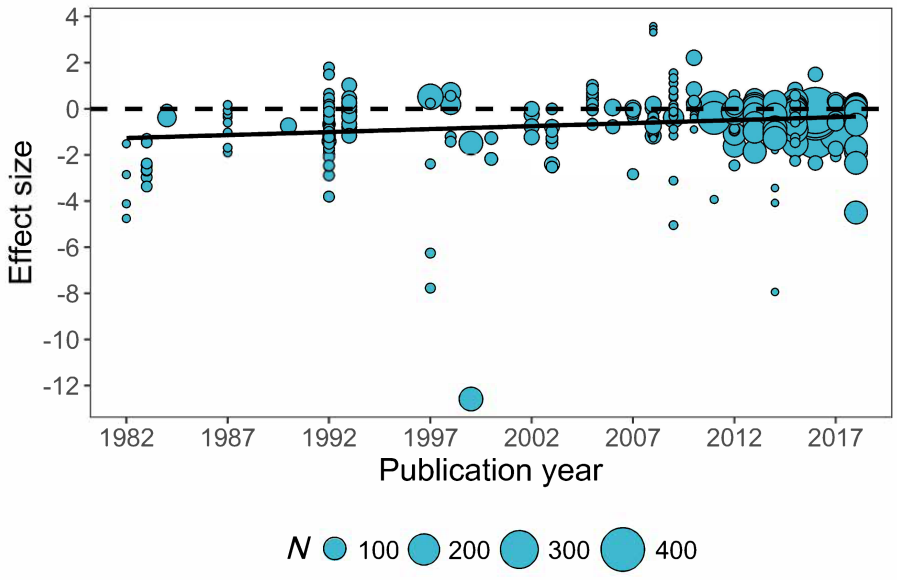


Figure 6. Bubble plot showing the correlation of publication year and effect size. The size of the circles indicates the sample size for each effect size calculated.

Discussion

Overall effect of nutrient limitation on ejaculate trait expression

Theory suggests that ejaculate trait expression should decrease when nutrients are limited, such that these traits should exhibit condition-dependent expression like that of other sexual traits. This is because male ejaculate traits can be under strong directional sexual selection due to their importance for male fitness, particularly under sperm competition, and selection is expected to promote costly exaggeration of at least some ejaculate traits (e.g., Linklater *et al.*, 2007; Crudgington *et al.*, 2009; Lüpold *et al.*, 2016; Godwin *et al.*, 2017). Although many studies have investigated the condition dependence of sperm and semen traits, variation in the nature and strength of responses remains poorly understood.

We carried out the first quantitative synthesis (a systematic review with a series of meta-analyses and meta-regression analyses), examining underlying sources of variation in responses of male ejaculate traits to nutrient limitation. Overall, the available empirical evidence indicates that male ejaculate traits show moderate but significant condition dependence (i.e., trait expression is reduced when males are nutrient limited). However, as expected, there is a high amount of heterogeneity in reported effects. Interestingly, differences between species (including both the phylogenetic signal and variation among species that is not explained by phylogeny) did not account for much of this variation. Rather, differences in observation-level effect sizes and between-study differences accounted for most of the variation in ejaculate responses to nutrient limitation, indicating that differences between other biologically relevant variables are modulating the effect of nutrient limitation on ejaculate trait expression. Through meta-regression analyses, we found that differences in the type of ejaculate trait, the type of nutrient limitation, and the ontogenetic life stage when nutrients were limited explained nearly 20% of this variation. Sub-analyses on arthropods, mammals, and fish also suggested some inter-taxon differences in ejaculate condition dependence, but the overall condition dependence of ejaculate traits appears to be a taxonomically widespread phenomenon. Therefore, while the prediction that male ejaculate traits are

condition dependent holds true overall, the strength of this response depends substantially on other biological variables.

Factors that explain variation in condition-dependent expression of ejaculate traits

We found that most ejaculate traits are likely to respond in a condition-dependent manner, but the strength of the response varied among traits, and some trait responses varied among taxa. Overall, seminal fluid quantity (i.e., non-sperm ejaculate size, and accessory gland size) decreased to the greatest extent with nutrient limitation, and seminal fluid quantity also showed substantial condition dependence in the sub-analyses on arthropods and mammals (we did not find any studies of seminal fluid quantity in fish). Sperm quantity also showed moderate condition dependence in arthropods and mammals (and approached a significant reduction in fish). This clear condition-dependent effect of nutrient limitation on sperm and seminal fluid quantity is consistent with expectations, given that selection can favour costly investment in these traits (e.g., Linklater *et al.*, 2007; Crudgington *et al.*, 2009). For example, the risk and intensity of sperm competition, as well as the likelihood of achieving a higher mating rate (normally in high-condition males) can favour increased expenditure on ejaculate production (Parker, 1982; Parker *et al.*, 1997; Gage & Morrow, 2003; Engqvist & Reinhold, 2005; Vahed & Parker, 2012; but see Simmons *et al.*, 2003). Additionally, testes size is correlated with sperm production (Parker *et al.*, 1997; Schärer, Ladurner & Rieger, 2004; reviewed in Simmons & Fitzpatrick, 2012). Therefore, the condition dependence of sperm quantity, at least partly, likely results from the production of larger testes by high-condition males, but it must be noted that testes size often scales hypo-allometrically with body size, which may result in low-condition males producing more sperm relative to body size.

By contrast, measures of sperm quality (i.e., sperm length, sperm movement and sperm normality) showed less-consistent condition-dependent responses. Sperm normality (proportion of sperm that are alive or morphologically normal) did not show a condition-dependent response overall, in arthropods, or in mammals, even though the proportion of live sperm has been shown to

be highly important in sperm competition and paternity, particularly in arthropods (e.g., Hunter & Birkhead, 2002; Fry & Wilkinson, 2004; Garcia-Gonzalez & Simmons, 2005). This suggests that, even in some taxa where selection acts on sperm viability, this does not necessarily exhibit strong condition dependence. Interestingly, sperm normality significantly decreased with nutrient limitation in fish, while sperm quantity did not, suggesting that selection may favour aspects of sperm quality over sperm quantity in fish. However, six out of the seven fish studies that measured sperm viability were on guppies (*Poecilia reticulata*), which are internal fertilisers. This suggests that guppies may experience stronger selection on sperm viability than do arthropods and mammals (see Fitzpatrick & Evans, 2014), but it is not clear whether this effect can be generalised across fish species, particularly to external fertilisers. Additionally, while sperm movement exhibited a small but significant reduction overall, and sperm length exhibited a small and near-significant reduction, there was substantial variation within and among taxa. Nutrient limitation strongly and significantly reduced sperm movement in mammals (albeit with substantial variation across studies) but did not significantly reduce sperm movement in fish, and nutrient limitation moderately reduced sperm length in fish but not in arthropods. This variation among taxa in trait responses to nutrient limitation likely reflects variation in sperm form and function, even between closely related species (reviewed in Reinhardt, Dobler & Abbott, 2015), potentially resulting from differential patterns of selection on sperm morphology, movement and viability among taxa (see Snook, 2005; Simmons & Fitzpatrick, 2012) – a question that warrants further research.

A reduction in total food quantity (i.e., reducing calories while maintaining nutrient ratios) resulted in a significant reduction in male ejaculate traits across taxa. Similarly, a reduction in ejaculate traits in response to protein limitation was observed in arthropods and mammals (the sample size was insufficient to test effects of protein limitation in fish). A reduction in total food quantity (i.e., calorie restriction) is known to induce condition-dependent responses in many reproductive traits and in male fitness (Kotiaho, 2000; Bonduriansky, 2007; Judge, Ting & Gwynne, 2008; Fritzsche & Arnqvist, 2015). However, the

effects of protein on male reproduction are less clear. Some recent studies have shown that effects of protein restriction on male fitness can be highly context dependent (e.g., Zajitschek *et al.*, 2012; Adler *et al.*, 2013; Macartney, Crean & Bonduriansky, 2017), and effects of protein restriction are generally less pronounced in males compared to females (e.g., Chippindale & Leroi, 1993; Adler *et al.*, 2013; Le Couteur *et al.*, 2016). However, our results suggest that protein limitation can indeed reduce male ejaculate trait expression across a wide range of taxa. Protein has been shown to be important for normal testicular functioning, with protein-deficient male rats producing a reduced quantity of sex hormones, atrophied accessory glands, and abnormal sperm (Srebnik & Nelson, 1962; Vawda & Mandlwana, 1990). Therefore, protein limitation is likely to have a significant effect on male fitness. Perhaps reported effects of dietary protein on male fitness are relatively subtle (e.g., Zajitschek *et al.*, 2012; Adler *et al.*, 2013; Macartney *et al.*, 2017) because these studies assayed male performance in the absence of sperm competition. Protein-restricted males might suffer reduced fitness if forced to compete for fertilisations against other males.

Overall, significant reductions in ejaculate trait expression were observed with nutrient limitation in both juveniles (i.e., before sexual maturity) and in adults, and differences in life stage did not explain much of the total variance in responses. However, there was some variation among taxonomic groups. In mammals, nutrient limitation in juveniles resulted in a significantly greater reduction in ejaculate traits compared to nutrient limitation in adults, and this also appeared to be the trend in arthropods. In comparison, juvenile nutrient limitation did not affect ejaculate trait expression in fish, but adult nutrient limitation resulted in a moderate and significant reduction in ejaculate traits. We predicted that adult nutrient limitation would reduce ejaculate trait expression because sperm and seminal products are produced by adults. This was the case overall, in arthropods and fish, and was nearing significance in mammals. However, it is interesting that juvenile nutrient limitation had an even stronger negative effect on ejaculate trait expression in arthropods and mammals, given that these traits are not yet fully developed in juveniles. This could reflect differences in how nutrients are mobilised in juveniles versus

adults (Gheorghe *et al.*, 2010) or variation in the ontogenetic timing and condition dependence of epigenetic programming of cells involved in the synthesis of sperm and seminal fluid (Macartney *et al.*, 2018a). For example, the developmental environment can alter many epigenetic factors such as DNA methylation, chromatin structure and non-coding RNAs (reviewed in Burdge & Lillycrop, 2010; Lo, Simpson & Sword, 2017), and such epigenetic factors have been shown to alter spermatogenesis (e.g., Song *et al.*, 2011; Wang *et al.*, 2017). Therefore, modifications of epigenetic factors in response to juvenile nutrient availability may alter the development and synthesis of adult ejaculate traits.

The condition dependence of ejaculate traits is relatively weak compared to body size

While many male ejaculate traits exhibited moderate levels of condition dependence, male body size showed a more than twofold greater response to nutrient limitation. Body size responses were also substantially more variable than those of ejaculate traits, and the inclusion of the moderator variables (degree of nutrient limitation, type of nutrient manipulation, and age at nutrient limitation) accounted for considerably more of this variation (>35%). Therefore, while many ejaculate traits are expected to co-vary with body size (e.g., Gage, 1994; Locatello *et al.*, 2008; O'Dea *et al.*, 2014; Macartney *et al.*, 2018b) these results suggest that body size and ejaculate traits respond somewhat differently to nutrient limitation, and that the condition dependence of ejaculate traits is weak compared to the condition dependence of overall growth. The condition dependence of ejaculate traits also appears to be relatively weak by comparison with that of many signal and weapon traits (Cotton *et al.*, 2004).

There are at least two plausible explanations for the relatively weak condition dependence of ejaculate traits (and apparent lack of condition dependence in some of these traits, such as sperm quality). One possibility is that sperm and semen traits are strongly canalised (buffered) against perturbations such as nutrient limitation (Wagner, Booth & Bagheri-Chaichian, 1997). While reproduction may still be possible even with substantial reduction in body size

and the expression of pre-copulatory secondary sexual traits (e.g., via sneak mating tactics), there may be a limit on the extent to which ejaculate quantity or quality can be reduced without suffering complete loss of reproductive capacity. Selection may therefore favour physiological mechanisms that maintain near-constant levels of resource allocation to ejaculate traits even when resources are limited in order to ensure that ejaculate quantity and quality exceed the minimum threshold levels required to achieve fertilisation even when males are in low condition.

Alternatively, body size may exhibit stronger condition dependence because overall growth requires a much greater investment of resources compared to the maintenance of ejaculate traits. In other words, ejaculate traits may exhibit weaker condition dependence because they are metabolically 'cheap' by comparison with body size (and perhaps also by comparison with many exaggerated signal and weapon traits). Discriminating between these contrasting hypotheses will require a better understanding of the metabolic costs of ejaculate trait expression.

Whatever its cause, the relatively weak condition dependence of ejaculate traits may limit the potential for the ejaculate to provide honest signals of male condition. If body size and pre-copulatory display traits are more strongly condition dependent than ejaculate traits, then these pre-copulatory traits would provide more honest signals of male mate quality (Andersson, 1982; Rowe & Houle, 1996). Therefore, selection may favour female preferences based on such pre-copulatory signals, rather than cryptic female mate choice based on ejaculate traits. However, if females are unable to exercise pre-copulatory mate choice (e.g., because males can coerce matings), selection could favour cryptic female mate choice based on the most condition-dependent ejaculate traits, such as sperm number (Eberhard & Cordero, 1995). While our results show that most ejaculate traits could serve as signals of environmental quality, it is less clear whether such traits could also serve as honest signals of genetic quality (although see Simmons & Kotiaho, 2002; Hosken *et al.*, 2003; Fisher *et al.*, 2006). If so, then sexual selection on ejaculate traits could contribute to the purging of deleterious mutations from

populations (Rowe & Houle, 1996; Agrawal, 2001; Lorch *et al.*, 2003), although the contribution of ejaculate traits to purging may be relatively weak by comparison with the role of pre-copulatory traits such as body size that often experience strong viability and sexual selection and exhibit strong condition dependence.

Publication bias and gaps in the literature

Our analyses suggest some publication bias and point to clear gaps in the literature. Trim-and-fill analyses suggested that some low-precision studies reporting positive effects of nutrient limitation on ejaculate traits (i.e., effects in the opposite direction to predictions) are missing from the published literature. This may be because studies that contradict expectations are less likely to be published, or because some heterogeneity in the data is not accounted for in our analyses (Nakagawa & Santos, 2012).

There are also many taxonomic gaps in the literature. Insects and mammals make up the bulk of studies that have manipulated diet and examined ejaculate traits, and rodents (mainly laboratory-strain rats and mice) comprised over 90% of the mammal studies. Therefore, we may be limited in our ability to draw general conclusions about ejaculate condition dependence, particularly given the large variation in sperm form and function among species (Snook, 2005; Simmons & Fitzpatrick, 2012; Reinhardt *et al.*, 2015).

Even within taxonomic groups, there are inconsistencies in the types of traits that are measured, and the types of diet manipulations applied. For example, in arthropods, very few studies have examined condition dependence of sperm movement (but see Macartney *et al.*, 2018b) and we did not find any studies that manipulated male condition by reducing carbohydrates. In mammals, we did not find any studies that measured sperm length. In fish, few studies have quantified testes size (but see Sullivan *et al.*, 2014; Mehlis *et al.*, 2015), we did not find any studies that measured ejaculate/accessory gland size, and most diet manipulations consisted of a reduction in total food quantity or a reduction in dietary carotenoids. Also, only 65% of studies included in our analyses reported an effect of nutrient limitation on body size as well as on

ejaculate traits. The need to report effects on body size in studies on the condition dependence of signal and weapon traits was emphasised by Cotton *et al.* (2004), and measuring effects on body size is equally important in studies of ejaculate traits.

Finally, we found that smaller effect sizes have been published in recent years. Surprisingly, the smaller effect sizes do not appear to be driven by sample size. This effect may be due to changes in research practices within the field (such as movements towards measuring sperm quality), or through a time-lag effect whereby the first papers to report an effect will publish larger effects compared to subsequent studies (Koricheva, Jennions & Lau, 2013).

Male nutrition and fitness

The available evidence suggests that nutrient-limited males often suffer reduced ejaculate quantity and sometimes quality. This pattern could have implications for our understanding of the evolution of mating systems. Low-condition males (which are typically smaller, and express reduced signal and weapon traits) are likely to be less successful in gaining access to females through combat or agonistic signalling (e.g., Rowe & Arnqvist, 1996; Sokolovska, Rowe & Johansson, 2000; Danielsson, 2001). In many species, low-condition males therefore employ alternative tactics such as sneaking or satellite behaviour, and such tactics are expected to enable low-condition males to achieve higher fitness than they would otherwise be able to achieve (e.g., Gross, 1996; Moczek & Emlen, 2000). However, if low-condition males are also disadvantaged in sperm competition relative to high-condition males because nutrient limitation depresses the quantity and/or quality of their sperm and seminal fluid, the reproductive potential of such males may be limited. For example, males that have fewer sperm are expected to be less competitive in 'raffle' competition based on sperm numbers (Parker, 1990), and a reduction in seminal fluid production can limit male mating rate (e.g., Reinhardt, Naylor & Siva-Jothy, 2011) and reduce female fecundity (reviewed in South & Lewis, 2011). However, in some species, low-condition males may be able to allocate sufficient resources to ejaculate traits to overcome such disadvantages. Moreover, males that are more likely to gain multiple matings (i.e., as a result

of their larger body size or exaggerated secondary sexual traits) may strategically allocate smaller ejaculates per mating in order to prevent ejaculate depletion. Such strategic ejaculate allocation has been demonstrated empirically in several species where it has been shown that larger males mate for a shorter time, transfer fewer sperm per mating, and may therefore fare poorly in sperm competition against smaller males (e.g., Rowe & Arnqvist, 1996; Danielsson, 2001; Fricke *et al.*, 2015; also see Pitnick, 1991). Unfortunately, our literature search found very few studies that measured the effect of nutrient limitation on the number of sperm transferred, so we were unable to test for differences in sperm production versus sperm transferred to the female based on nutrient availability. This would be especially interesting to address in the future.

Additionally, conditional tactics can be complex and subtle, and more research is needed to understand the nature of such tactics at the post-copulatory stage. Different ejaculate traits may interact to affect the outcome of sperm competition. For example, Lüpold *et al.* (2012) found that male *Drosophila melanogaster* that had slower-swimming sperm were at a competitive advantage when competing against males with faster sperm as the slow-sperm males produced longer sperm and transferred more sperm per mating. The slow sperm were then more likely to remain in the female sperm storage organ to be used for fertilisation. However, these interactions may be taxon specific as other studies have found positive correlations between sperm swimming speed and fitness under sperm competition (e.g., Birkhead *et al.*, 1999; Gasparini *et al.*, 2010; Boschetto *et al.*, 2011). Moreover, pre- and post-copulatory ejaculate traits could interact in their effects on male fitness. Tazzyman *et al.* (2009) used a model to show that the fitness effect of reduced ejaculate production under nutrient limitation could depend on the energetic cost and likelihood of gaining matings. Thus, our understanding of the condition dependence of male fitness would be enhanced by studies that consider both pre- and post-copulatory competitive environments, investigate how the condition dependence of body size interacts with the condition dependence of ejaculate traits to affect male reproductive success, and explore the specific roles of various ejaculate traits in the tactics employed by low- and high-condition males under sperm competition.

Conclusions

1) As predicted by theory, we show that most male ejaculate traits exhibit condition-dependent expression by reducing trait expression when males are nutrient limited, and this effect is conserved across broad taxonomic groups. The literature reports highly variable ejaculate responses, and we show that variation among ejaculate traits, and in the type of nutrient limitation and the ontogenetic life stage when nutrients are limited, jointly account for 19% of this variation.
2) We show that sperm and semen quantity are moderately and consistently condition dependent in fish, mammals and arthropods. By contrast, while some aspects of sperm quality are also condition dependent, this effect is less consistent across taxa.
3) A reduction in total food quantity and protein induce the strongest condition-dependent responses. Nutrient limitation at the adult stage affects male ejaculate traits across all major taxa. Nutrient limitation at the juvenile stage significantly affects ejaculate trait expression in arthropods and mammals, but not in fish, and nutrient limitation at the adult stage significantly affects ejaculate trait expression in arthropods and fish, but not in mammals.
4) The condition dependence of male ejaculate traits was relatively weak compared with that of body size. This could reflect canalisation (buffering) of male ejaculate traits. Alternatively, the metabolic costs of ejaculate trait expression may be relatively low by comparison with those of body size. The relatively weak condition dependence of ejaculate traits limits their ability to serve as honest signals of male quality.
5) A reduction in sperm and semen traits as well as in body size is likely to reduce male fitness, and these effects could interact. Future studies should aim to examine effects of both nutrient limitation and pre- and post-copulatory competition on male fitness (i.e., progeny sired).
6) There are considerable gaps in the literature. Future studies should investigate ejaculate condition dependence in other taxonomic groups (especially birds, reptiles, and non-rodent mammals), as well as the condition dependence of less-studied traits within certain taxa (i.e.,

sperm movement in arthropods, sperm length in mammals, and semen traits in fish). Future studies should ensure they report effects on body size as well as on the ejaculate trait of interest.

References

References identified with asterisks were included in the meta-analyses.

ADLER, M.I., CASSIDY, E.J., FRICKE, C. & BONDURIANSKY, R. (2013). The lifespan-reproduction trade-off under dietary restriction is sex-specific and context-dependent. *Experimental Gerontology* **48**, 539–548.

AGRAWAL (2001). Transgenerational consequences of plant responses to herbivory: an adaptive maternal effect? *The American Naturalist* **157**, 555–569.

*ALAVI, S.M.H., PŠENIČKA, M., POLICAR, T., RODINA, M., HAMÁČKOVÁ, J., KOZÁK, P. & LINHART, O. (2009). Sperm quality in male *Barbus barbus* L. fed different diets during the spawning season. *Fish Physiology and Biochemistry* **35**, 683–693.

*AMITIN, E.G. & PITNICK, S. (2007). Influence of developmental environment on male- and female-mediated sperm precedence in *Drosophila melanogaster*. *Journal of Evolutionary Biology* **20**, 381–391.

ANDERSSON, M. (1982). Sexual selection, natural selection, and quality advertisement. *Biological Journal of the Linnean Society* **17**, 375–393.

AVILA, F.W., SIROT, L.K., LAFLAMME, B.A., RUBINSTEIN, C.D. & WOLFNER, M.F. (2011). Insect seminal fluid proteins: identification and function. *Annual Review of Entomology* **56**, 21–40.

*AYDILEK, N., VARISLI, O., KOCYIGIT, A., TASKIN, A. & KAYA, M.S. (2011). Effect of dietary restriction on sperm characteristic and oxidative status on testicular tissue in young rats exposed to long-term heat stress. *Andrologia* **2**, 1055–1061.

*BAILEY, A.M., LEGAN, S.J. & DEMAS, G.E. (2017). Exogenous kisspeptin enhances seasonal reproductive function in male Siberian hamsters. *Functional Ecology* **31**, 1220–1230.

*BAKER, R.H., DENNIFF, M., FUTERMAN, P., FOWLER, K., POMIANKOWSKI, A. & CHAPMAN, T. (2003). Accessory gland size influences time to sexual maturity and mating frequency in the stalk-eyed fly, *Cyrtodiopsis dalmanni*. *Behavioral Ecology* **14**, 607–611.

BEAUSOLEIL, J.M.J., DOUCET, S.M., HEATH, D.D. & PITCHER, T.E. (2012). Spawning coloration, female choice and sperm competition in the redside dace, *Clinostomus elongatus*. *Animal Behaviour* **83**, 969–977.

BIRKHEAD, T.R., MARTÍNEZ, J.G., BURKE, T. & FROMAN, D.P. (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society B: Biological Sciences* **266**, 1759–1764.

*BLANK, J.L. & DESJARDINS, C. (1984). Spermatogenesis is modified by food intake. *Biology of Reproduction* **30**, 410–415.

*BLAY, S. & YUVAL, B. (1997). Nutritional correlates of reproductive success of male Mediterranean fruit flies (Diptera: Tephritidae). *Animal Behaviour* **54**, 59–66.

BOGGS, C.L. (1981). Nutritional and life-history determinants of resource-allocation in holometabolous insects. *American Naturalist* **117**, 692–709.

BONDURIANSKY, R. (2007). The evolution of condition-dependent sexual dimorphism. *American Naturalist* **169**, 9–19.

BOSCHETTO, C., GASPARINI, C. & PILASTRO, A. (2011). Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology* **65**, 813–821.

*BRINKWORTH, M.H., ANDERSON, D. & MCLEAN, A.E.M. (1992). Effects of dietary imbalances on spermatogenesis in CD-1 mice and CD rats. *Food and Chemical Toxicology* **30**, 29–35.

BROMFIELD, J.J. (2014). Seminal fluid and reproduction: much more than previously thought. *Journal of Assisted Reproduction and Genetics* **31**, 627–636.

BUNNING, H., RAPKIN, J., BELCHER, L., ARCHER, C.R., JENSEN, K. & HUNT, J. (2015). Protein and carbohydrate intake influence sperm number and fertility in male cockroaches, but not sperm viability. *Proceedings of the Royal Society B: Biological Sciences* **282**, 1–8.

BURDGE, G.C. & LILLYCROP, K.A. (2010). Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. *Annual Reviews of Nutrition* **30**, 315–339.

*BURRIS, Z.P. & DAM, H.G. (2015). Spermatophore production as a function of food abundance and age in the calanoid copepods, *Acartia tonsa* and *Acartia hudsonica*. *Marine Biology* **162**, 841–853.

CAMERON, E., DAY, T. & ROWE, L. (2007). Sperm competition and the evolution of ejaculate composition. *American Naturalist* **169**, 158–172.

CHAMBERLAIN, S.A., HOVICK, S.M., DIBBLE, C.J., RASMUSSEN, N.L., VAN ALLEN, B.G., MAITNER, B.S., AHERN, J.R., BELL-DERESKE, L.P., ROY,

C.L., MEZA-LOPEZ, M., CARRILLO, J., SIEMANN, E., LAJEUNESSE, M.J. & WHITNEY, K.D. (2012). Does phylogeny matter? Assessing the impact of phylogenetic information in ecological meta-analysis. *Ecology Letters* **15**, 627–636.

*CHAPIN, R.E., GULATI, D.K., BARNES, L.H. & TEAGUE, J.L. (1993). The effects of feed restriction on reproductive function in Sprague-Dawley rats. *Fundamental and Applied Toxicology* **20**, 23–29.

CHAPMAN, T. (2001). Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity* **87**, 511–521.

*CHINOY, N.J., MEHTA, D. & JHALA, D.D. (2006). Effects of fluoride ingestion with protein deficient or protein enriched diets on sperm function of mice. *Fluoride* **39**, 11–16.

CHIPPINDALE, A. & LEROI, A. (1993). Phenotypic plasticity and selection in *Drosophila* life-history evolution. I. Nutrition and the cost of reproduction. *Journal of Evolutionary Biology* **6**, 171–193.

COHEN, J. (1988). *Statistical Power Analysis for the Behavioral Sciences,* 2nd Edition. Erlbaum, Hillsdale, NJ.

*COSTA, A.M., ANJOS-DUARTE, C.S., RORIZ, A.K.P., DIAS, V.S. & JOACHIM-BRAVO, I.S. (2012). Male diet and age influence to inhibit female remating in *Ceratitis capitata* (Diptera: Tephritidae). *Journal of Applied Entomology* **136**, 456–463.

COTTON, S., FOWLER, K. & POMIANKOWSKI, A. (2004). Do sexual ornaments demonstrate heightened condition-dependent expression as predicted by the handicap hypothesis? *Proceedings of the Royal Society B: Biological Sciences* **271**, 771–783.

CREAN, A.J., ADLER, M.I. & BONDURIANSKY, R. (2016). Seminal fluid and mate choice: new predictions. *Trends in Ecology & Evolution* **31**, 253–255.

CRUDGINGTON, H.S., FELLOWS, S., BADCOCK, N.S. & SNOOK, R.R. (2009). Experimental manipulation of sexual selection promotes greater male mating capacity but does not alter sperm investment. *Evolution* **63**, 926–938.

DANIELSSON, I. (2001). Antagonistic pre- and ejaculate sexual selection on male body size in a water strider (*Gerris lacustris*). *Proceedings of the Royal Society B: Biological Sciences* **268**, 77–81.

DARWIN, C. (1859). *On the origin of species by means of natural selection*. London, United Kingdom.

DARWIN, C. (1871). *The Descent of Man and Selection in Relation to Sex*. John Murry, London, UK.

*DAVIES, S., CROS, T., RICHARD, D., MEDDLE, S.L., TSUTSUI, K. & DEVICHE, P. (2015). Food availability, energetic constraints and reproductive development in a wild seasonally breeding songbird. *Functional Ecology* **29**, 1421–1434.

*DÁVILA, F. & ARON, S. (2017). Protein restriction affects sperm number but not sperm viability in male ants. *Journal of Insect Physiology* **100**, 71–76.

*DEMAS, G.E. & NELSON, R.J. (1998). Photoperiod, ambient temperature, and food availability interact to affect reproductive and immune function in adult male deer mice *Peromyscus maniculatus*). *Journal of Biological Rhythms* **13**, 253–262.

*DEVIGILI, A., KELLEY, J.L., PILASTRO, A. & EVANS, J.P. (2013). Expression of pre- and postcopulatory traits under different dietary conditions in guppies. *Behavioral Ecology* **24**, 740–749.

DEWSBURY, D.A. (1982). Ejaculate cost and male choice. *American Naturalist* **119**, 601–610.

*DOLATPANAH, M.B., TOWHIDI, A., FARSHAD, A., RASHIDI, A. & REZAYAZDI, A. (2008). Effects of dietary fish oil on semen quality of goats. *Asian-Australasian Journal of Animal Sciences* **21**, 29–34.

*DRONEY, D.C. (1998). The influence of the nutritional content of the adult male diet on testis mass, body condition and courtship vigour in a Hawaiian *Drosophila*. *Functional Ecology* **12**, 920–928.

*DUPLOUY, A., WOESTMANN, L., GALLEGO ZAMORANO, J. & SAASTAMOINEN, M. (2017). Impact of male condition on his spermatophore and consequences for female reproductive performance in the Glanville fritillary butterfly. *Insect Science* **25**, 1–13.

DUVAL, S. & TWEEDIE, R. (2000). Trim and fill: a simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. *Biometrics* **56**, 455–463.

EBERHARD, W.G. & CORDERO, C. (1995). Sexual selection by cryptic female choice on male seminal products – a new bridge between sexual selection

and reproductive physiology. *Trends in Ecology & Evolution* **10**, 493–496.

*EDMONDS, K.E., RIGGS, L. & STETSON, M.H. (2003). Food availability and photoperiod affect reproductive development and maintenance in the marsh rice rat (*Oryzomys palustris*). *Physiology and Behavior* **78**, 41–49.

EGGER, M., SMITH, G.D., SCHNEIDER, M. & MINDER, C. (2015). Bias in meta-analysis detected by a simple, graphical test. *British Medical Journal* **14**, 1–16.

EMLEN, D.J. (1997). Diet alters male horn allometry in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society B: Biological Sciences* **264**, 567–574.

*ENGQVIST, L. (2008). Genetic variance and genotype reaction norms in response to larval food manipulation for a trait important in scorpionfly sperm competition. *Functional Ecology* **22**, 127–133.

ENGQVIST, L., DEKOMIEN, G., LIPPMANN, T., EPPLEN, J.T. & SAUER, K.P. (2007). Sperm transfer and paternity in the scorpionfly *Panorpa cognata*: large variance in traits favoured by ejaculate episodes of sexual selection. *Evolutionary Ecology* **21**, 801–816.

ENGQVIST, L. & REINHOLD, K. (2005). Pitfalls in experiments testing predictions from sperm competition theory. *Journal of Evolutionary Biology* **18**, 116–123.

*EVANS, J.P., LYMBERY, R.A., WIID, K.S., RAHMAN, M. & GASPARINI, C. (2017). Sperm as moderators of environmentally induced paternal effects in a livebearing fish. *Biology Letters* **13**, 10–13.

*EVANS, J.P., RAHMAN, M.M. & GASPARINI, C. (2015). Genotype-by-environment interactions underlie the expression of pre- and ejaculate sexually selected traits in guppies. *Journal of Evolutionary Biology* **28**, 959–972.

*FERKAU, C. & FISCHER, K. (2006). Costs of reproduction in male *Bicyclus anynana* and *Pieris napi* butterflies: effects of mating history and food limitation. *Ethology* **112**, 1117–1127.

FISHER, D.O., DOUBLE, M.C., BLOMBERG, S.P., JENNIONS, M.D. & COCKBURN, A. (2006). Post-mating sexual selection increases lifetime fitness of polyandrous females in the wild. *Nature* **444**, 89–92.

FITZPATRICK, J.L. & EVANS, J.P. (2014). Postcopulatory inbreeding avoidance

in guppies. *Journal of Evolutionary Biology* **27**, 2585–2594.

FRICKE, C., ADLER, M.I., BROOKS, R.C. & BONDURIANSKY, R. (2015). The complexity of male reproductive success: effects of nutrition, morphology, and experience. *Behavioral Ecology* **26**, 617–624.

*FRITZSCHE, K. & ARNQVIST, G. (2015). The effects of male phenotypic condition on reproductive output in a sex role-reversed beetle. *Animal Behaviour* **102**, 209–215.

FRY, C.L. & WILKINSON, G.S. (2004). Sperm survival in female stalk-eyed flies depends on seminal fluid and meiotic drive. *Evolution* **58**, 1622–1626.

GAGE, M.J.G. (1994). Associations between body size, mating pattern, testis size and sperm lengths across butterflies. *Proceedings of the Royal Society B: Biological Sciences* **258**, 247–254.

GAGE, M.J.G. & MORROW, E.H. (2003). Experimental evidence for the evolution of numerous, tiny sperm via sperm competition. *Current Biology* **13**, 754-757.

GARCIA-GONZALEZ, F. & SIMMONS, L.W. (2005). The evolution of polyandry: intrinsic sire effects contribute to embryo viability. *Journal of Evolutionary Biology* **18**, 1097–1103.

*GASPARINI, C., DEVIGILI, A., DOSSELLI, R. & PILASTRO, A. (2013). Pattern of inbreeding depression, condition dependence, and additive genetic variance in Trinidadian guppy ejaculate traits. *Ecology and Evolution* **3**, 4940–4953.

GASPARINI, C., SIMMONS, L.W., BEVERIDGE, M. & EVANS, J.P. (2010). Sperm swimming velocity predicts competitive fertilization success in the green swordtail *Xiphophorus helleri*. *PLoS ONE* **5**, 1–5.

GHEORGHE, C., GOYAL, R., MITTAL, A. & LONGO, L. (2010). Gene expression in the placenta: maternal stress and epigenetic responses. *International Journal of Developmental Biology* **54**, 507–523.

GODWIN, J.L., VASUDEVA, R., MICHALCZYK, L., MARTIN, O.Y., LUMLEY, A.J., CHAPMAN, T. & GAGE, M.J.G. (2017). Experimental evolution reveals that sperm competition intensity selects for longer, more costly sperm. *Evolution Letters* **1**, 102–113.

GOMENDIO, M., MARTIN-COELLO, J., CRESPO, C., MAGANA, C. & ROLDAN, E.R.S. (2006). Sperm competition enhances functional capacity of

mammalian spermatozoa. *Proceedings of the National Academy of Sciences* **103**, 15113–15117.

GROSS, M.R. (1996). Alternative reproductive strategies and tactics: diversity within sexes. *Trends in Ecology & Evolution* **11**, 92–98.

*GWYNNE, D.T. (1990). Testing parental investment and the control of sexual selection in katydids: the operational sex ratio. *American Naturalist* **136**, 474–484.

GWYNNE, D.T. (1993). Food quality controls sexual selection in Mormon crickets by altering male mating investment. *Ecology* **74**, 1406–1413.

HADFIELD, J. (2010). MCMC methods for multi-response generalized linear mixed models: The {MCMCglmm} {R} Package}. *Journal of Statistical Software* **33**, 1–22.

*HAMDI, S.A., NASSIF, O.I. & ARDAWI, M.S.M. (1997). Effect of marginal or severe dietary zinc deficiency on testicular development and functions of the rat. *Archives of Andrology* **38**, 243–253.

HEINO, M. & KAITALA, V. (1999). Evolution of resource allocation between growth and reproduction in animals with indeterminate growth. *Journal of Evolutionary Biology* **12**, 423–429.

*HELFENSTEIN, F., LOSDAT, S., MOLLER, A.P., BLOUNT, J.D. & RICHNER, H. (2010). Sperm of colourful males are better protected against oxidative stress. *Ecology Letters* **13**, 213–222.

*HELLRIEGEL, B. & BLANCKENHORN, W.U. (2002). Environmental influences on the gametic investment of yellow dung fly males. *Evolutionary Ecology* **16**, 505–522.

HIGGINS, J.P.T., THOMPSON, S.G., DEEKS, J.J. & ALTMAN, D.G. (2003). Measuring inconsistency in meta-analyses. *British Medical Journal* **327**, 557–560.

*HONDA, K., TAKASE, H. & ÔMURA, H. (2012). Procurement of exogenous ammonia by the swallowtail butterfly, *Papilio polytes*, for protein biosynthesis and sperm production. *Naturwissenschaften* **99**, 695–703.

HOSKEN, D.J., GARNER, T.W.J., TREGENZA, T., WEDELL, N. & WARD, P.I. (2003). Superior sperm competitors sire higher-quality young. *Proceedings of the Royal Society B: Biological Sciences* **270**, 1933–1938.

HOTHORN, T., BRETZ, F. & WESTFALL, P. (2008). Simultaneous inference in

general parametric models. *Biometrical Journal* **50**, 346–363.

HUNTER, F.M. & BIRKHEAD, T.R. (2002). Sperm viability and sperm competition in insects. *Current Biology* **12**, 121–123.

IMMLER, S., PITNICK, S., PARKER, G.A., DURRANT, K.L., LÜPOLD, S., CALHIM, S. & BIRKHEAD, T.R. (2011). Resolving variation in the reproductive tradeoff between sperm size and number. *Proceedings of the National Academy of Sciences* **108**, 5325–5330.

*JIA, Z., JIANG, Z. & SAKALUK, S.K. (2000). Nutritional condition influences investment by male katydids in nuptial food gifts. *Ecological Entomology* **25**, 115–118.

JUDGE, K.A., TING, J.J. & GWYNNE, D.T. (2008). Condition dependence of male lifespan and calling effort in a field cricket. *Evolution* **62**, 868–878.

*KAHRL, A.F. & COX, R.M. (2015). Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behavioral Ecology* **26**, 1502–1511.

*KALDUN, B. & OTTI, O. (2016). Condition-dependent ejaculate production affects male mating behavior in the common bedbug *Cimex lectularius*. *Ecology and Evolution* **6**, 2548–2558.

*KATSUKI, M., OKADA, Y. & OKADA, K. (2012). Impacts of diet quality on life-history and reproductive traits in male and female armed beetle, *Gnatocerus cornutus*. *Ecological Entomology* **37**, 463–470.

*KELLY, C.D. & GWYNNE, D.T. (2016). The effect of condition on mate searching speed and copulation frequency in the Cook Strait giant weta. *Behavioral Ecology and Sociobiology* **70**, 1403–1409.

*KOMATSU, H., KAKIZOE, T., NIIJIMA, T., KAWACHI & SUGIMURA, T. (1982). Increased sperm abnormalities due to dietary restriction Hideki. *Mutation Research* **93**, 439–446.

KORICHEVA, J., JENNIONS, M.D. & LAU, J. (2013). Temporal trends in effect sizes: causes, detection and implications. In *Handbook of Meta-analysis in Ecology and Evolution* (eds J. KORICHEVA, J. GUREVITCH & K. MENGERSEN), pp. 237–254. Princeton University Press, Princeton.

KOTIAHO, J.S. (2000). Testing the assumptions of conditional handicap theory: costs and condition dependence of a sexually selected trait. *Behavioral Ecology and Sociobiology* **48**, 188–194.

*KUMARI, D., NAIR, N. & BEDWAL, R.S. (2011). Effect of dietary zinc deficiency on testes of Wistar rats: morphometric and cell quantification studies. *Journal of Trace Elements in Medicine and Biology* **25**, 47–53.

*LAMBROT, R., XU, C., SAINT-PHAR, S., CHOUNTALOS, G., COHEN, T., PAQUET, M., SUDERMAN, M., HALLETT, M. & KIMMINS, S. (2013). Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. *Nature Communications* **4**, 1–13.

LAMUNYON, C.W. & SAMUEL, W. (1999). Evolution of sperm size in nematodes: sperm competition favours larger sperm. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **266**, 263–267.

LE COUTEUR, D.G., SOLON-BIET, S., COGGER, V.C., MITCHELL, S.J., SENIOR, A., DE CABO, R., RAUBENHEIMER, D. & SIMPSON, S.J. (2016). The impact of low-protein high-carbohydrate diets on aging and lifespan. *Cellular and Molecular Life Sciences* **73**, 1237–1252.

*LEDERHOUSE, A.R.C., AYRES, M.P., SCRIBER, J.M., LEDERHOUSE, R.C. & AYRES, M.P. (1990). Adult nutrition affects male virility in *Papilio glaucus* L. *Functional Ecology* **4**, 743–751.

*LEWIS, Z., SASAKI, H. & MIYATAKE, T. (2011). Sex starved: do resource-limited males ensure fertilization success at the expense of precopulatory mating success? *Animal Behaviour* **81**, 579–583.

LIAO, W.B., HUANG, Y., ZENG, Y., ZHONG, M.J., LUO, Y. & LÜPOLD, S. (2018). Ejaculate evolution in external fertilizers: influenced by sperm competition or sperm limitation? *Evolution* **72**, 4–17.

LINKLATER, J.R., WERTHEIM, B., WIGBY, S. & CHAPMAN, T. (2007). Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. *Evolution* **61**, 2027–2034.

LO, N., SIMPSON, S. & SWORD, G. (2017). Epigenetics and developmental plasticity in orthopteroid insects. *Current Opinion in Insect Science* **3**, 1–10.

LOCATELLO, L., RASOTTO, M.B., ADRIAENSSENS, B. & PILASTRO, A. (2008). Ejaculate traits in relation to male body size in the eastern mosquitofish *Gambusia holbrooki*. *Journal of Fish Biology* **73**, 1600–1611.

LOCATELLO, L., RASOTTO, M.B., EVANS, J.P., PILASTRO, A. (2006). Colourful male guppies produce faster and more viable sperm . *Journal of Evolutionary*

Biology **19**, 1595–1602.

*LOPUCH, S. & RADWAN, J. (2009). Condition dependence of sexual attractiveness in the bank vole. *Behavioral Ecology and Sociobiology* **63**, 339–344.

LORCH, P., PROULX, S., ROWE, L. & DAY, T. (2003). Condition-dependent sexual selection can accelerate adaptation. *Evolutionary Ecology Research* **5**, 867–881.

LÜPOLD, S., MANIER, M.K., BERBEN, K.S., SMITH, K.J., DALEY, B.D., BUCKLEY, S.H., BELOTE, J.M. & PITNICK, S. (2012). How multivariate ejaculate traits determine competitive fertilization success in *Drosophila melanogaster*. *Current Biology* **22**, 1667–1672.

LÜPOLD, S., MANIER, M.K., PUNIAMOORTHY, N., SCHOFF, C., STARMER, W.T., LUEPOLD, S.H.B., BELOTE, J.M. & PITNICK, S. (2016). How sexual selection can drive the evolution of costly sperm ornamentation. *Nature* **533**, 535–538.

LÜPOLD, S. & PITNICK, S. (2018). Sperm form and function: what do we know about the role of sexual selection? *Reproduction* **155**, 229–243.

MACARTNEY, E.L., CREAN, A.J. & BONDURIANSKY, R. (2017). Adult dietary protein has age- and context-dependent effects on male ejaculate performance. *Journal of Evolutionary Biology* **38**, 42–49.

MACARTNEY, E.L., CREAN, A.J. & BONDURIANSKY, R. (2018*a*). Epigenetic paternal effects as costly, condition-dependent traits. *Heredity* **121**, 248–256.

*MACARTNEY, E.L., NICOVICH, P.R., BONDURIANSKY, R. & CREAN, A.J. (2018*b*). Developmental diet irreversibly shapes male ejaculate traits in the neriid fly *Telostylinus angusticollis*. *Journal of Evolutionary Biology* **31**, 1894–1902.

MARCOTTE, M., DELISLE, J. & MCNEIL, J.N. (2007). Effects of different male remating intervals on the reproductive success of *Choristoneura rosaceana* males and females. *Journal of Insect Physiology* **53**, 139–145.

MAUTZ, B.S., MØLLER, A.P. & JENNIONS, M.D. (2013). Do male secondary sexual characters signal ejaculate quality? A meta-analysis. *Biological Reviews* **88**, 669–682.

*MCFARLAND, C.A., TALENT, L.G., QUINN, M.J., BAZAR, M.A., WILBANKS,

M.S., NISANIAN, M., GOGAL, R.M., JOHNSON, M.S., PERKINS, E.J. & GUST, K.A. (2012). Multiple environmental stressors elicit complex interactive effects in the western fence lizard (*Sceloporus occidentalis*). *Ecotoxicology* **21**, 2372–2390.

*MCGRAW, L.A., FIUMERA, A.C., RAMAKRISHNAN, M., MADHAVARAPU, S., CLARK, A.G. & WOLFNER, M.F. (2007). Larval rearing environment affects several ejaculate traits in *Drosophila melanogaster*. *Biology Letters* **3**, 607–610.

*MEHLIS, M., RICK, I.P. & BAKKER, T.C.M. (2015). Dynamic resource allocation between pre- and postcopulatory episodes of sexual selection determines competitive fertilization success. *Proceedings of the Royal Society B: Biological Sciences* **282**, 1–9.

*MELO, M.C., ALMEIDA, F.R.C.L., CALDEIRA-BRANT, A.L., PARREIRA, G.G. & CHIARINI-GARCIA, H. (2014). Spermatogenesis recovery in protein-restricted rats subjected to a normal protein diet after weaning. *Reproduction, Fertility and Development* **26**, 787–796.

*MERSON, M.H., KIRKPATRICK, R.L., SCANLON, P.F. & GWAZDAUSKAS, F.C. (1983). Influences of restricted food-intake on reproductive characteristics and carcass fat of male white-footed mice. *Journal of Mammology* **64**, 353–355.

MOCZEK, A.P. & EMLEN, D.J. (2000). Male horn dimorphism in the scarab beetle, *Onthophagus taurus*: do alternative reproductive tactics favour alternative phenotypes? *Animal Behaviour* **59**, 459–466.

MOLLER, A.P. & DELOPE, F. (1994). Differential costs of a secondary sexual character – an experimental test of the handicap principle. *Evolution* **48**, 1676–1683.

MOREHOUSE, N.I. (2014). Condition-dependent ornaments, life histories, and the evolving architecture of resource-use. *Integrative and Comparative Biology* **54**, 591–600.

NAKAGAWA, S., NOBLE, D.W.A., SENIOR, A.M. & LAGISZ, M. (2017). Meta-evaluation of meta-analysis: ten appraisal questions for biologists. *BMC Biology* **15**, 1–14.

NAKAGAWA, S. & SANTOS, E.S.A. (2012). Methodological issues and advances in biological meta-analysis. *Evolutionary Ecology* **26**, 1253–1274.

NAKAGAWA, S. & SCHIELZETH, H. (2013). A general and simple method for obtaining R^2 from generalized linear mixed-effects models. *Methods in Ecology and Evolution* **4**, 133–142.

*NELSON, R.J., KITA, M., BLOM, J.M.C. & RHYNEGREY, J. (1992). Photoperiod influences among the critical caloric-intake necessary to maintain reproduction among male deer mice (*Peromyscus maniculatus*). *Biology of Reproduction* **42**, 226–232.

NOBLE, D.W.A., LAGISZ, M., O'DEA, R.E. & NAKAGAWA, S. (2017). Nonindependence and sensitivity analyses in ecological and evolutionary meta-analyses. *Molecular Ecology* **26**, 1–16.

NUR, N. & HASSON, O. (1984). Phenotypic plasticity and the handicap principle. *Journal of Theoretical Biology* **110**, 275–297.

*O'BRIEN, S. & HAU, M. (2005). Food cues and gonadal development in neotropical spotted antbirds (*Hylophylax naevioides*). *Journal of Ornithology* **146**, 332–337.

*O'DEA, R.E., JENNIONS, M.D. & HEAD, M.L. (2014). Male body size and condition affects sperm number and production rates in mosquitofish, *Gambusia holbrooki*. *Journal of Evolutionary Biology* **27**, 2739–2744.

OLSSON, M.J., MADSEN, T. & SHINE, R. (1997). Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proceedings of the Royal Society B: Biological Sciences* **264**, 455–459.

*OMU, A.E., AL-AZEMI, M.K., AL-MAGHREBI, M.A., MATHEW, C.T., OMU, F.E., KEHINDE, E.O., ANIM, J.T., ORIOWO, M.A. & MEMON, A. (2015). Molecular basis for the effects of zinc deficiency on spermatogenesis: an experimental study in the Sprague-dawley rat model. *Indian Journal of Urology* **31**, 57–64.

PARKER, G.A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biological Reviews* **45**, 535–567.

PARKER, G.A., BALL, M., STOCKLEY, P. & GAGE, M. (1997). Sperm competition games: a prospective analysis of risk assessment. *Proceedings of the Royal Society B: Biological Sciences* **264**, 1793–1802.

PARKER, G.A. (1982). Why are there so many tiny sperm – sperm competition and the maintenance of two sexes. *Journal of Theoretical Biology* **96**, 281–294.

PARKER, G.A. (1990). Sperm competition games: raffles and roles. *Proceedings of*

the Royal Society B: Biological Sciences **242**, 120–126.

PARKER, G.A. & BALL, M.A. (2005). Sperm competition, mating rate and the evolution of testis and ejaculate sizes: a population model. *Biology letters* **1**, 235–238.

*PERFITO, N., KWONG, J.M.Y., BENTLEY, G.E. & HAU, M. (2008). Cue hierarchies and testicular development: is food a more potent stimulus than day length in an opportunistic breeder (*Taeniopygia g. guttata*)? *Hormones and Behavior* **53**, 567–572.

*PERRY, J.C. & ROWE, L. (2010). Condition-dependent ejaculate size and composition in a ladybird beetle. *Proceedings of the Royal Society B: Biological Sciences* **277**, 3639–3647.

PERRY, J.C., SIROT, L. & WIGBY, S. (2013). The seminal symphony: how to compose an ejaculate. *Trends in Ecology & Evolution* **28**, 414–422.

PERRY, J.C. & TSE, C.T. (2013). Extreme costs of mating for male two-spot ladybird beetles. *PLoS ONE* **8**, 1–5.

PITNICK, S. (1991). Male size influences mate fecundity and remating interval in *Drosophila melanogaster*. *Animal Behaviour* **41**, 735–745.

POIANI, A. (2006). Complexity of seminal fluid: a review. *Behavioral Ecology and Sociobiology* **60**, 289–310.

*PROCTOR, A.H.C. (1992). Effect of food deprivation on mate searching and spermatophore production in male water mites (Acari: Unionicolidae). *Functional Ecology* **6**, 661–665.

RADWAN, J. (1996). Intraspecific variation in sperm competition success in the bulb mite: a role for sperm size. *Proceedings of the Royal Society B: Biological Sciences* **263**, 855–859.

*RAHMAN, M., TURCHINI, G.M., GASPARINI, C., NORAMBUENA, F. & EVANS, J.P. (2014). The expression of pre- and postcopulatory sexually selected traits reflects levels of dietary stress in guppies. *PLoS ONE* **9**, 1–12.

*RAHMAN, M.M., GASPARINI, C., TURCHINI, G.M. & EVANS, J.P. (2015). Testing the interactive effects of carotenoids and polyunsaturated fatty acids on ejaculate traits in the guppy *Poecilia reticulata* (Pisces: Poeciliidae). *Journal of Fish Biology* **86**, 1638–1643.

*RAHMAN, M.M., KELLEY, J.L. & EVANS, J.P. (2013). Condition-dependent expression of pre- and postcopulatory sexual traits in guppies. *Ecology and*

Evolution **3**, 2197–2213.

RAMM, S., PARKER G., STOCKLEY, P. (2005). Sperm competition and the evolution of male reproductive anatomy in rodents. *Proceedings of the Royal Society B: Biological Sciences* **272**, 949–959.

*REHM, S., WHITE, T.E., ZAHALKA, E.A., STANISLAUS, D.J., BOYCE, R.W. & WIER, P.J. (2008). Effects of food restriction on testis and accessory sex glands in maturing rates. *Toxicologic Pathology* **36**, 687–694.

REINHARDT, K., DOBLER, R. & ABBOTT, J. (2015). An ecology of sperm: sperm diversification by natural selection. *Annual Review of Ecology, Evolution, and Systematics* **46,** 457–459.

REINHARDT, K., NAYLOR, R. & SIVA-JOTHY, M.T. (2011). Male mating rate is constrained by seminal fluid availability in bedbugs, *Cimex lectularius*. *PLoS ONE* **6**, 1–8.

ROWE, L. & ARNQVIST, G. (1996). Analysis of the causal components of assortative mating in water striders. *Behavioral Ecology and Sociobiology* **38**, 279–286.

ROWE, L. & HOULE, D. (1996). The lek paradox and the capture of genetic variance by condition dependent traits. *Proceedings of the Royal Society B: Biological Sciences* **263**, 1415–1421.

ROWE, M., ALBRECHT, T., CRAMER, E.R.A., JOHNSEN, A., LASKEMOEN, T., WEIR, J.T. & LIFJELD, J.T. (2015). Postcopulatory sexual selection is associated with accelerated evolution of sperm morphology. *Evolution* **69**, 1044–1052.

SCHÄRER, L., LADURNER, P. & RIEGER, R.M. (2004). Bigger testes do work more: experimental evidence that testis size reflects testicular cell proliferation activity in the marine invertebrate, the free-living flatworm *Macrostomum* sp. *Behavioral Ecology and Sociobiology* **56**, 420–425.

SCHIELZETH, H. (2010). Simple means to improve the interpretability of regression coefficients. *Methods in Ecology and Evolution* **1**, 103–113.

SENIOR, A.M., GRUEBER, C., KAMIYA, T., LAGISZ, M., O'DWYER, K., SANTOS, E.S.A. & NAKAGAWA, S. (2016). Heterogeneity in ecological and evolutionary meta-analyses: its magnitude and implications. *Statistical Reports* **97**, 576–582.

*SIMMONS, L.W. (2012). Resource allocation trade-off between sperm quality

and immunity in the field cricket, *Teleogryllus oceanicus*. *Behavioral Ecology* **23**, 168–173.

SIMMONS, L.W. & FITZPATRICK, J.L. (2012). Sperm wars and the evolution of male fertility. *Reproduction* **144**, 519–534.

SIMMONS, L.W. & KOTIAHO, J.S. (2002). Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution* **56**, 1622–1631.

*SIMMONS, L.W., TEALE, R.J., MAIER, M., STANDISH, R.J., BAILEY, W.J. & WITHERS, P.C. (1992). Some costs of reproduction for male bushcrickets, *Requena verticalic* (Orthoptera: Tettigoniidae) allocating resources to mate attraction and nuptial feeding. *Behavioral Ecology and Sociobiology* **31**, 57–62.

SIMMONS, L.W., TOMKINS, J.L. & HUNT, J. (1999). Sperm competition games played by dimorphic male beetles. *Proceedings of the Royal Society B: Biological Sciences* **266**, 145–150.

SIMMONS, L.W., WERNHAM, J., GARCÍA-GONZÁLEZ, F. & KAMIEN, D. (2003). Variation in paternity in the field cricket *Teleogryllus oceanicus*: no detectable influence of sperm numbers or sperm length. *Behavioral Ecology* **14**, 539–545.

*SITZMANN, B.D., LEONE, E.H., MATTISON, J.A., INGRAM, D.K., ROTH, G.S., URBANSKI, H.F., ZELINSKI, M.B. & OTTINGER, M.A. (2010). Effects of moderate calorie restriction on testosterone production and semen characteristics in young rhesus macaques (*Macaca mulatta*). *Biology of Reproduction* **83**, 635–640.

SNOOK, R.R. (2005). Sperm in competition: not playing by the numbers. *Trends in Ecology and Evolution* **20**, 46–53.

SOKOLOVSKA, N., ROWE, L. & JOHANSSON, F. (2000). Fitness and body size in mature odonates. *Ecological Entomology* **25**, 239–248.

SONG, N., LIU, J., AN, S., NISHINO, T., HISHIKAWA, Y. & KOJI, T. (2011). Immunohistochemical analysis of histone H3 modifications in germ cells during mouse spermatogenesis. *Acta Histochemica Et Cytochemica* **44**, 183–190.

SOUTH, A. & LEWIS, S.M. (2011). The influence of male ejaculate quantity on female fitness: a meta-analysis. *Biological Reviews* **86**, 299–309.

*SPEARS, N. & CLARKE, J.R. (1987). Effect of nutrition, temperature and

photoperiod on the rate of sexual maturation of the field vole (*Microtus agrestis*). *Journal of Reproduction and Fertility* **80**, 175–181.

SREBNIK, H.H. & NELSON, M.M. (1962). Anterior pituitary function in male rats deprived of dietary protein. *Endocrinology* **70**, 723–730.

*SULLIVAN, M., BROWN, A.C. & CLOTFELTER, E.D. (2014). Dietary carotenoids do not improve motility or antioxidant capacity in cichlid fish sperm. *Fish Physiology and Biochemistry* **40**, 1399–1405.

TAZZYMAN, S.J., PIZZARI, T., SEYMOUR, R.M. & POMIANKOWSKI, A. (2009). The evolution of continuous variation in ejaculate expenditure strategy. *The American Naturalist* **174**, 71–82.

*TOMÁŠEK, O., ALBRECHTOVÁ, J., NĚMCOVÁ, M., OPATOVÁ, P. & ALBRECHT, T. (2017). Trade-off between carotenoid-based sexual ornamentation and sperm resistance to oxidative challenge. *Proceedings of the Royal Society of London B: Biological Sciences* **284**, 1–9.

TOURMENTE, M., GOMENDIO, M. & ROLDAN, E.R.S. (2011). Sperm competition and the evolution of sperm design in mammals. *BMC Evolutionary Biology* **11**, 1–10.

VAHED, K., PARKER, G.A. (2012). The evolution of large testes: sperm competition or male mating rate? *Ethology* **118**, 107–117.

VAWDA, A.I. & MANDLWANA, J.G. (1990). The effects of dietary protein deficiency on rat testicular function. *Andrologia* **22**, 575–583.

*VEGA-TREJO, R., JENNIONS, M.D. & HEAD, M.L. (2016). Are sexually selected traits affected by a poor environment early in life? *BMC Evolutionary Biology* **16**, 1–12.

VELLNOW, N., MARIE-ORLEACH, L., ZADESENETS, K.S. & SCHÄRER, L. (2018). Bigger testes increase paternity in a simultaneous hermaphrodite, independently of the sperm competition level. *Journal of Evolutionary Biology* **31**, 180–196.

*VERMEULEN, A., ENGELS, S. & SAUER, K.P. (2008). Maintenance of variance in sperm transfer rates in a scorpionfly: food availability, genetic basis, and heritability. *Behavioral Ecology and Sociobiology* **63**, 77–83.

VIECHTBAUER, W. (2010). Conducting meta-analyses in R with the metafor package. *Journal of Statistical Software* **36**, 1–48.

WAGNER, G.P., BOOTH, G. & BAGHERI-CHAICHIAN, H. (1997). A population

genetic theory of canalization. *Evolution* **51**, 329–347.

WALLACE, B., SMALL, K., BRODLEY, C., LAU, J. & TRIKALINOS, T. (2012). Deploying an interactive machine learning system in an evidence-based practice center: abstrackr. *Proceedings of the ACM International Health Informatics Symposium* **1**, 819–824.

WANG, L., XU, Z., KHAWAR, M.B., LIU, C. & LI, W. (2017). The histone codes for meiosis. *Reproduction* **154**, 65–79.

*WATANABE, M. & HIROTA, M. (1999). Effects of sucrose intake on spermatophore mass produced by male swallowtail butterfly *Papilio xuthus* L. *Zoological Science* **16**, 55–61.

*WATANABE, M. & KAMIKUBO, M. (2005). Effects of saline intake on spermatophore and sperm ejaculation in the male swallowtail butterfly *Papilio xuthus* (Lepidoptera : Papilionidae). *Entomological Science* **8**, 161–166.

WEDELL, N., GAGE, M.J.G. & PARKER, G.A. (2002). Sperm competition, male prudence and sperm-limited females. *Trends in Ecology & Evolution* **17**, 313–320.

WILLIAMS, P., DAY, T. & CAMERON, E. (2005). The evolution of sperm-allocation strategies and the degree of sperm competition. *Evolution* **59**, 492–499.

*WILSON, D.E., FELT, K.D., CAMPBELL, E.N., COHEN, O.R., GEISEL, L.E., GRAHAM, J. & PALIULIS, L. V (2016). Resource allocation in food-restricted male *Physocyclus mexicanus* Banks, 1898 spiders does not favor proportionally larger testes (Araneae: Pholcidae). *Journal of Arachnology* **44**, 408–411.

*YOSSA, R., SARKER, P.K., PROULX, É & VANDENBERG, G.W. (2015). The effects of the dietary biotin on zebrafish *Danio rerio* reproduction. *Aquaculture Research* **46**, 117–130.

ZAJITSCHEK, F., LAILVAUX, S.P., DESSMANN, J. & BROOKS, R. (2012). Diet, sex, and death in field crickets. *Ecology and Evolution* **2**, 1627–1636.

Supplementary material

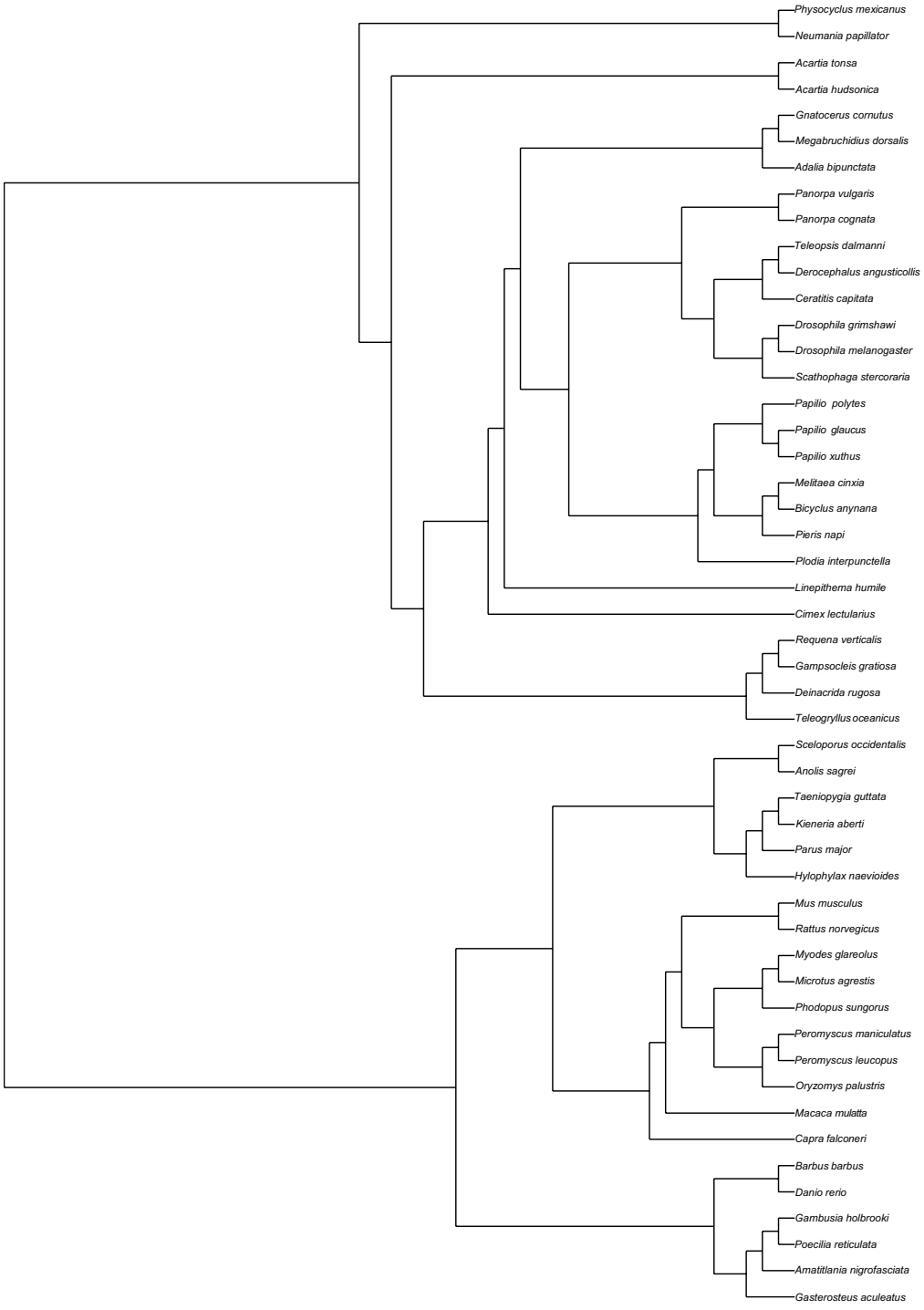


Figure S1. Phylogenetic tree of the species included in the meta-analysis. Note that some species names differ from those used in their papers of origin due to recent taxonomic revisions, in the Open Tree of Life.

Appendix S1

Factorial tables

The numbers in the factorial tables represent the number of effect sizes (*k*) and the number of studies (*N*) within each taxonomic group that correspond to the different moderator variables. Note that the sum of the entries per table differ due to complexities within study designs and lack of reporting of ontogenetic life stage in some studies. Many studies measured multiple traits which increased the number of entries per trait. Also, some studies manipulated multiple dietary components separately within the experimental design, and some studies did not report the age at which nutrients were manipulated. These complexities were accounted for in the analyses by including animal group ID as a random effect.

Table S1. Factorial table showing the number of effect sizes (*k*) and the number of studies (*N*) across taxonomic groups that reported effects on nutrient limitation on post-copulatory traits.

	Arachnids		Birds		Crustaceans		Fish		Insects		Mammals		Reptiles	
	k	*N*	*k*	*N*	*k*	*N*	*k*	*N*	*k*	*N*	*k*	*N*	*k*	*N*
Body size	2	1	2	2	0	0	14	10	21	13	30	15	2	2
Sperm length	0	1	1	0	0	0	12	7	6	3	0	0	1	1
Testes/spermatophore	2	2	3	3	8	2	3	2	30	18	37	16	2	1
Sperm movement	0	0	2	2	0	0	24	12	2	1	10	6	3	1
Sperm number	0	0	0	0	0	0	26	13	25	11	33	11	6	1
Sperm viability	0	0	1	1	0	0	7	6	6	3	30	8	0	0
Seminal fluid quantity	0		0	0	0	0	0	0	15	6	26	9	0	0

Table S2. Factorial table showing the number of effect sizes (k) and the number of studies (N) per diet manipulation (this includes body size k).

	Arachnids		Birds		Crustaceans		Fish		Insects		Mammals		Reptiles	
	k	*N*	*k*	*N*	*k*	*N*	*k*	*N*	*k*	*N*	*k*	*N*	*k*	*N*
Carbohydrates	0	0	0	0	0	0	0	0	0	0	8	1	0	0
Fats	0	0	0	0	0	0	9	2	0	0	13	2	0	0
Micronutrients	0	0	0	0	0	0	22	1	21	4	26	7	0	0
Protein	0	0	0	0	0	0	24	1	31	11	37	4	0	0
Quality	0	0	6	3	0	0	0	5	9	4	0	0	0	0
Quantity	4	2	4	2	8	2	37	9	44	13	102	14	14	2

Table S3. Factorial table showing the number of effect sizes (*k*) and the number of studies (*N*) across taxa and the ontogenetic life stage when nutrients were manipulated.

	Arachnids		Birds		Crustaceans		Fish		Insects		Mammals		Reptiles	
	k	*N*	*k*	*N*	*k*	*N*	*k*	*N*	*k*	*N*	*k*	*N*	*k*	*N*
Juvenile	3	1	0	0	0	0	38	3	36	11	36	6	12	1
Adult	1	1	10	4	8	2	41	7	69	22	118	11	2	1

Appendix S2

Analyses accounting for phylogenetic relatedness

Table S4. Post-copulatory traits with phylogenetic relatedness included as a random effect. SMD, standardised mean difference; CI, credible interval; *k*, number of effect sizes.

Moderator	Factor	SMD	95%CI	*k*
Overall post-copulatory response		–0.496	–0.854, –0.138	348
Degree of nutrient limitation		0.012	–0.181, 0.202	253
Diet	Quantity	–0.659	–0.912, –0.405	164
	Protein	–0.391	–0.742, –0.041	83
	Micronutrients	–0.413	–0.843, 0.016	65
	Fat	–0.127	–0.704, 0.451	20
	Quality	–0.048	–0.493, 0.396	8
Traits	Seminal fluid quantity	–0.854	–1.234, –0.173	27
	Spermatophore/testes size	–0.653	–0.960, –0.345	85
	Sperm quantity	–0.482	–0.796, –0.17	90
	Sperm length	–0.409	–0.882, 0.064	20
	Sperm movement	–0.329	–0.717, 0.059	41
	Sperm normality	0.085	–0.302, 0.473	44
Life stage	Juvenile	–0.589	–0.879, –0.298	101
	Adult	–0.428	–0.646, –0.210	212
	Juvenile–adult comparison	–0.161	–0.469, 0.148	

Heterogeneity (I^2) = 90.021%; moderator variables (degree of nutrient restriction, diet, traits and life stage) account for 18.983% (R^2) of variance.

Table S5. Body size with phylogenetic relatedness included as a random effect. SMD, standardised mean difference; CI, credible interval; *k*, number of effect sizes.

	Factor	SMD	95%CI	*k*
Overall body size response		–1.287	–2.374, –0.121	71
Degree of nutrient limitation		–0.044	–0.471, 0.383	44
Diet	Quantity	–1.514	–3.085, 0.056	47
	Protein	–1.026	–2.868. 0.817	9
	Micronutrients	0.085	–2.077, 2.248	8
Life stage	Juvenile	–1.521	–2.724, –0.318	25
	Adult	–1.398	–2.538, –0.258	37
	Juvenile–adult comparison	–0.161	–0.469, 0.147	

Heterogeneity (I^2) = 97.317%; moderator variables (degree of nutrient restriction, diet, and life stage) account for 12.407% (R^2) of variance.

Appendix S3

Results with outlier (Chinoy et al., 2006) excluded.

While we excluded Chinoy *et al.* (2006) from the publication bias analyses due to their extremely large reported effects of nutrient limitation, the exclusion of this paper from the post-copulatory trait meta-analysis, meta-regression and mammal sub-analyses do not alter outcomes due to its low weight in the models (i.e., due to a small sample size).

Meta-analysis

SMD [outlier excluded] = –0.531; CI = –0.690, –0.369; I^2= 87.660. Different experimental animal groups explained 22.43% of the variance, species accounted for 9.66% of the variance, and differences between effect size ID (i.e., observation-level random effect) accounted for 55.56% of the variance.

Meta-regression

There was little difference between the meta-regression output with and without Chinoy *et al.* (2006) included in the analyses (Fig. S2). The only slight change is that exclusion of their study caused credible intervals to fall below 0 for sperm length, making the reduction in sperm length significant, and the mean effect of nutrient limitation on sperm viability shifted from positive to negative, but the effects of nutrient limitation on sperm viability were still small and non-significant.

The inclusion of all moderator variables in a full model accounted for 15.01% of the variance in response.

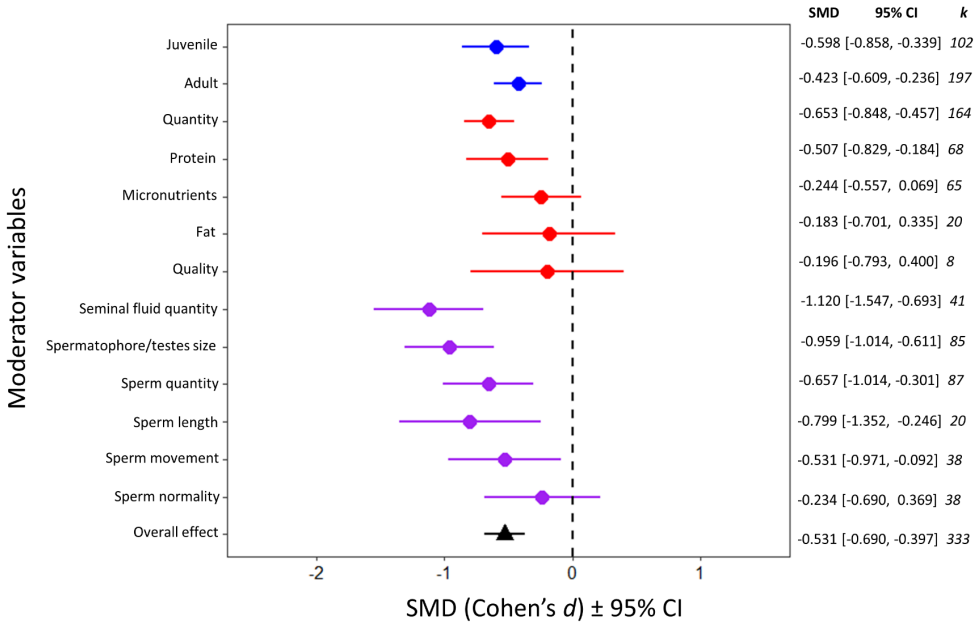


Figure S2. Forest plot displaying the overall effect (black triangle) of a reduced diet on male post-copulatory traits when Chinoy *et al.* (2006) is excluded from the analysis. Effects of ontogenetic life stage are in blue, type of diet manipulation in red, and the effects on different post-copulatory traits in purple. Text on the right displays the standardised mean difference (SMD, Cohen's *d*), 95% credible interval (CI), and the number of observations for each moderator variable (*k*).

Mammal sub-analysis

Sub-analyses on mammals do not change when Chinoy *et al.* (2006) is removed from the data. The only difference is that the direction of the mean effect of nutrient limitation on sperm viability changes from positive to negative, but the effect of diet is still small and non-significant. Also, the difference in post-copulatory reduction when nutrients are limited during development *versus* as adults becomes marginally significant (SMD [$\text{mammal}_{\text{adult–juvenile comparison}}$] = –0.711; CI = –1.459, –0.036) (Fig. S3).

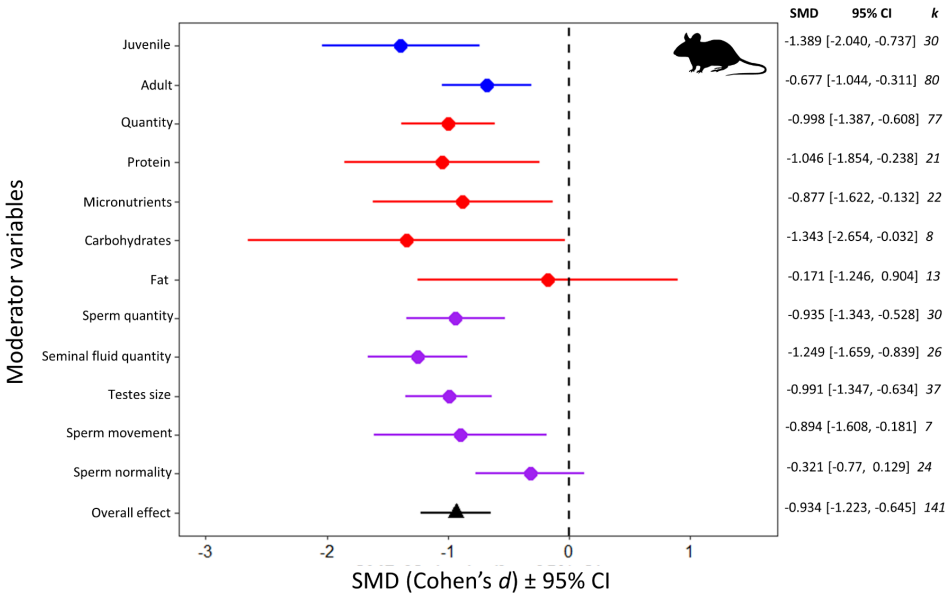


Figure S3. Forest plot displaying the overall effect (black triangle) of a reduced diet on male post-copulatory traits in mammals when Chinoy *et al*. (2006) is excluded from the analysis. The effects of ontogenetic life stage are in blue, the type of diet manipulation in red, and the effects on different post-copulatory traits in purple. Text displays the standardised mean difference (SMD, Cohen's *d*), 95% credible interval (CI), and the number of observations for each moderator variable (*k*).

Appendix S4

Meta-analysis and meta-regression on male body size

Does nutrient limitation cause an overall decrease in male body size?

In the parallel meta-analysis on the same study individuals, a decrease in nutrient intake resulted in a large reduction in body size (SMD [total] = –1.359; CI = –1.779, –0.940) (Fig. S4), suggesting that body size is substantially more condition-dependent compared to post-copulatory traits. Including phylogenetic relatedness reduced model fit (ΔAIC = 3.111), phylogenetic heritability was very low (Pagel's Lambda <0.001%), and results remained robust (see Appendix S2), so it was not included as a random effect in the model. Body size had a much higher amount of heterogeneity in responses to nutrient limitation (I^2[total] = 96.5%), with differences between experimental animal groups accounting for 75.8% of the variance, differences between species that were not accounted for by phylogenetic relatedness accounting for 19.6% of the variance, and effect size ID accounting for 1.1% of the variance. The high amount of heterogeneity in the response of male body size to nutrient limitation suggests that other factors modulate the responses of such traits to a reduction in nutrient intake. Therefore, we attempted to explain this variation using meta-regression analyses of several moderator variables: the degree of nutrient reduction, the life-stage at which nutrients were reduced, and the type of nutrient manipulation (Fig. S4). Overall, these moderators explained 35.5% (R^2) of the variance in responses of body size to nutrient limitation.

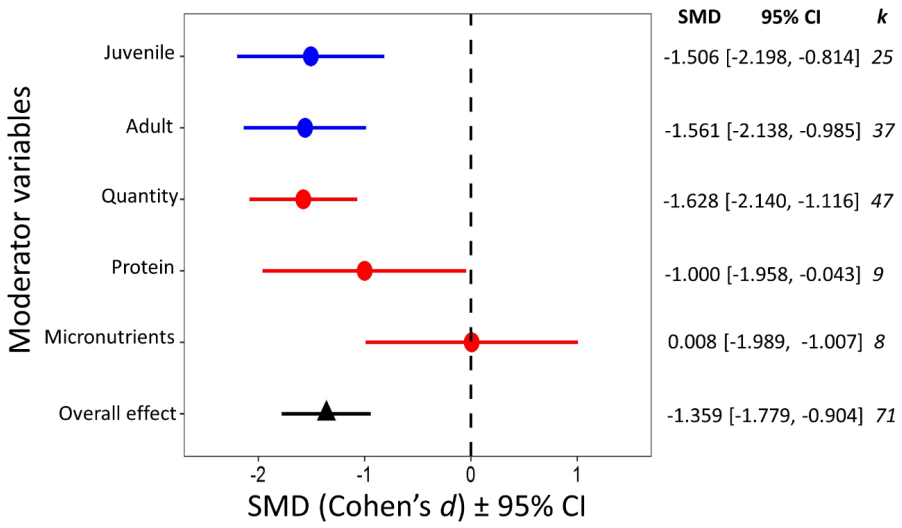


Figure S4. Forest plot displaying the overall effect (black triangle) of nutrient limitation on male body size, as well as the effects of ontogenetic life stage (blue), and type of diet manipulation (red). Text on the right displays the standardised mean difference (SMD, Cohen's *d*), 95% credible interval (CI), and the number of observations for each moderator variable (*k*).

Taxonomic sub-analyses of nutrient restriction at different life-stages on male body size

The effects of nutrient limitation on body size should be taxon specific due to differences in growth patterns between broad taxonomic groups. We completed sub-analyses on arthropods, mammals and fish to test for differences in the effect of nutrient limitation as juveniles and adults. As would be expected, juvenile nutrient limitation in arthropods resulted in a large and significant decrease in male body size and a reduction in adult nutrients resulted in a small, non-significant decrease in body size, and this difference was significant (SMD [arthropods $_{juvenile}$] = –1.691; CI = –2.677; k = 11, –0.703; SMD[arthropods $_{adult}$] = –0.785; CI = –1.766, 0.1955; k = 10]. This difference was significant (SMD [arthropods $_{adult–juvenile\ comparison}$] = –2.594; CI = –4.786, –0.403). In mammals, nutrient limitation during development and as adults resulted in large and significant decreases in male body size (SMD [mammal $_{juvenile}$] = –2.377; CI = –3.943, –0.812; k = 6; SMD [mammal $_{adult}$] = –2.719; CI = –3.732, –1.705]; k = 15), and there was no difference between life stages (SMD [mammal $_{adult–juvenile\ comparison}$] = –2.036; CI = –2.036, 1.121). By contrast, adult nutrient limitation had a very large, significant effect on male

body size in fish (SMD [fish $_{juvenile}$] = –0.207; CI = –2.287, 1.874; k = 5; SMD [fish $_{adult}$] = –2.207; CI = –4.179, –0.236]; k = 9), but there was no significant difference between life stages (SMD [fish $_{adult–juvenile\ comparison}$] = 1.793; CI = –2.269, 5.856).

Taxonomic sub-analyses on the effects of specific nutrients on body size

Unfortunately, we were unable to complete taxonomic sub-analyses of the effects of different nutrient manipulations on body size as the number of observations within each diet category was too small (k < 5). However, we had no *a priori* predictions that specific types of diet manipulations would cause a greater or smaller body size decrease depending on taxonomic group.

Publication bias

Statistical assessment of our data, after controlling for heterogeneity, suggests that there is substantial publication bias on the effects of nutrient limitation on male body size (Eggers regression on the meta-analytic residuals: β [intercept] = –1.666, S.E. = 0.352, P = <0.001). This was confirmed with the Eggers regression test for funnel asymmetry (t_{58} = –1.735, P_{MCMC} = <0.001), and the 'trim-and-fill' method added 11 points to the right side (positive effects) of the funnel plot (Fig. S5). However, this is likely to be due to a real biological phenomenon as it is unlikely that nutrient limitation would cause a substantial increase in male body size.

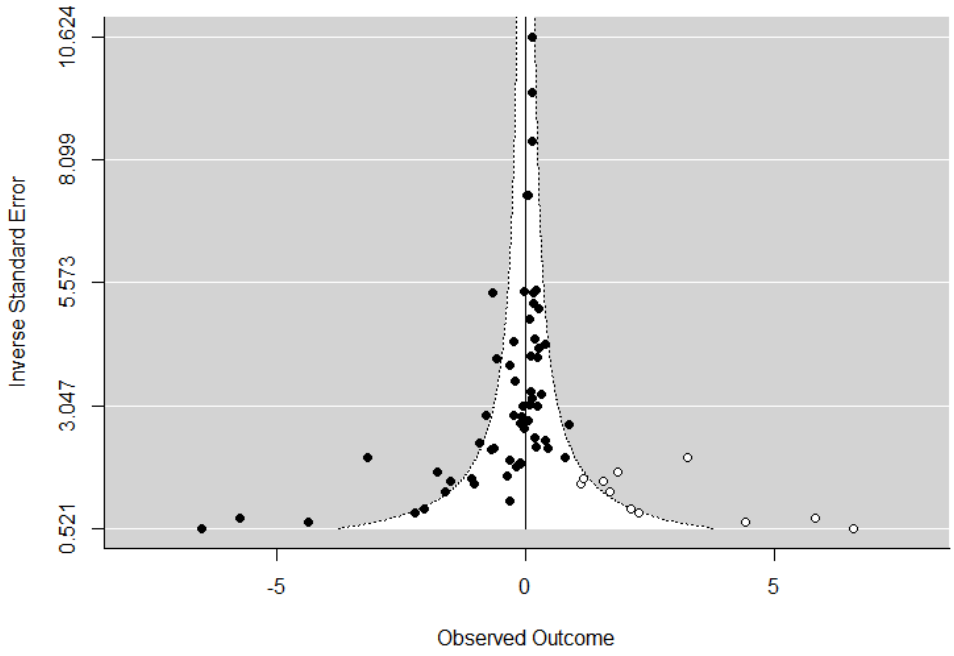


Figure S5. Trim-and-fill funnel plot to test for publication bias in reported effects of nutrient limitation on male body size. The *x*-axis indicates the meta-analytic residuals, and the *y*-axis indicates the inverse standard error (precision). The black dots represent published data, and the white dots represent 'missing' data as calculated by the trim-and-fill analysis.

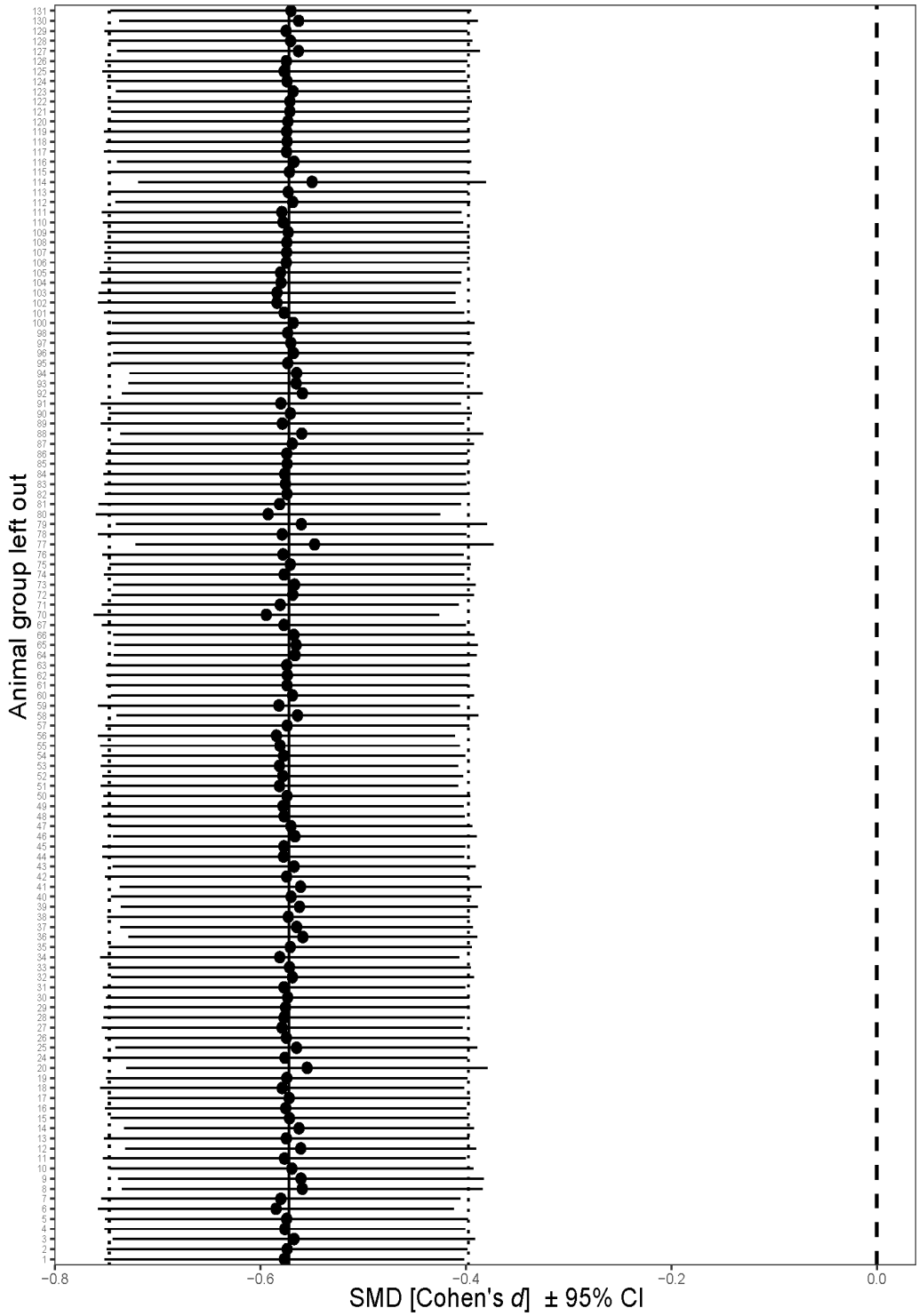


Figure S6. 'Leave one group out' sensitivity analysis on ejaculate traits.

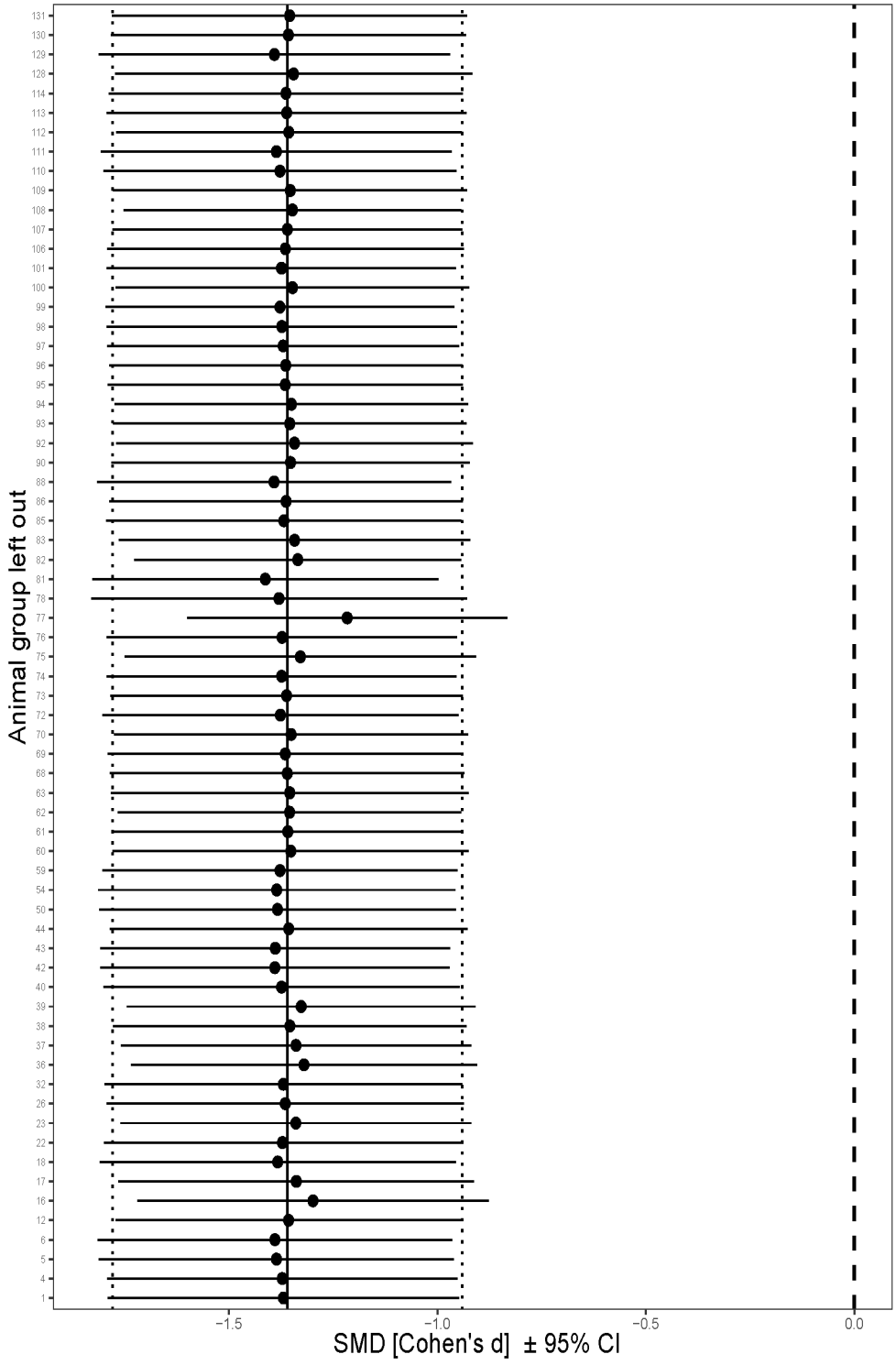


Figure S7. 'Leave one group out' sensitivity analysis on body size.

Chapter Three

DEVELOPMENTAL DIET IRREVERSIBLY SHAPES MALE POST-COPULATORY TRAITS IN THE NERIID FLY *TELOSTYLINUS ANGUSTICOLLIS*

Erin L Macartney, Philip R Nicovich, Russell Bonduriansky, Angela J Crean

Journal of Evolutionary Biology (2018). ***31****, 1894–1902*

ELM and AJC conceived and designed the experiment; ELM ran the experiment and collected the data; AJC and PRN developed the computational method for measuring sperm movement within the female reproductive tract; AJC implemented the computational method for converting sperm movement videos into Hz. ELM analysed and wrote the manuscript; RB and AJC provided valuable advice throughout analysis and writing.

Abstract

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

Introduction

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

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

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

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

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

Methods

Experimental set-up

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

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

On the day of adult emergence, males from each developmental diet were randomly assigned to either a 'nutrient-rich' ("R") adult diet of brown sugar and yeast, or a 'nutrient-poor' ("P") adult diet of brown sugar only, in a fully crossed design with four diet combinations (RR, RP, PR, PP) where flies had *ad libitum* access to the developmental and adult diet provided. This adult dietary manipulation (which limits protein but not carbohydrates) differs from the developmental manipulation (which limits both protein and carbohydrates) because *T. angusticollis* has substantially different nutrient requirements in developmental versus adult stages. Developing larvae require a relatively balanced mixture of protein and carbohydrates (Sentinella *et al.*, 2013; Runagall-McNaull *et al.*, 2015). By contrast, adult males require carbohydrates to survive, but can survive and reproduce with minimal dietary protein (Adler *et al.*, 2013; Macartney *et al.*, 2017). Nonetheless, protein is a key nutrient for male reproduction in many species (Droney, 1998; Baker *et al.*, 2003; Costa *et al.*, 2012), and we have previously found context-dependent effects of adult dietary protein on male reproductive performance in *T. angusticollis* (Adler *et al.*, 2013; Macartney *et al.*, 2017). Males were housed with 5 other males per cage to promote investment in post-copulatory traits (but only one male from each cage was included in the study). They were not housed with any females in order to ensure the males remained virgins before the sperm movement assay.

The mean age of experimental males was 23.3 ± 9.64 (SD) days at the time of the assay. This is a relatively young age as male neriid flies can live up to 150 days in the laboratory (Hooper *et al.*, 2017). Cages were lined with cocopeat and watered daily to provide hydration. Two separate blocks of the experiment were completed (block 1: N=51; block 2: N=52, with n = 11-15 individuals/treatment combination/block).

Sperm movement within the female reproductive tract

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

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

Male Morphological measurements

Flies were photographed at 6.5x magnification to measure thorax length as a measure of body size (Bonduriansky, 2007), and testes and three types of accessory-glands ('epitesticular', 'lobate', and 'tubular'; (Fig. S1)) were dissected on to a slide moistened with saline solution and photographed at 25x

magnification. Testes were then severed to release sperm and a coverslip placed over the sample. Five to eight sperm per male were photographed at 400x magnification and a mean taken. Thorax length, testes and accessory glands were photographed using a Zeiss AxioCamERc5s camera mounted to a Zeiss Stemi 2000-CS microscope, and sperm were photographed using a Zeiss AxioCam HSc camera mounted on a Zeiss AxioScope A1 compound microscope (Zeiss, Jena, Germany). Thorax length, testes area, accessory-gland area, and sperm length measurements were obtained from images using ImageJ software (Rasband, 2015). Each trait was measured twice, and the average value was used in analyses to reduce measurement error.

Statistical analysis

All analyses were completed in R version 3.3.2, using the lme4 (Bates *et al.*, 2015) and LmerTest (Kuznetsova, Brockhoff, & Rune, 2017) packages. Linear mixed effects models (Gaussian distributions) were used to test main effects and interactions of developmental and adult diets on all male post-copulatory traits with block as a random effect. LmerTest calculates p-values and degrees of freedom for Gaussian mixed models based on the Satterthwaite approximation for denominator degrees of freedom (Schaalje, McBride, & Fellingham, 2002). Thorax length was centred within developmental diet treatment groups because the developmental diet affects thorax length, making the diet treatment partially redundant with thorax length, and centred thorax length was included as a co-variate in the models to test for correlations of relative body size and trait size within developmental diets. Male age was initially included as a co-variate in all post-copulatory trait models but was later removed from the models, except sperm length, as its inclusion did not significantly improve model fit (based on likelihood ratio tests (LRTs) with a conservative cut-off of $p>0.1$) (Table S1 for LRTs). Sperm movement was analysed with two large outliers removed (both males reared on a nutrient-rich developmental diet with sperm movement > 4 standard deviations above the mean). Removal of these outliers makes our results more conservative. Sperm length was analysed with one outlier removed, and the epitesticular gland was analysed with one outlier removed. Outliers were identified as being greater than four standard deviations away from the mean.

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

Results

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

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

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

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

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

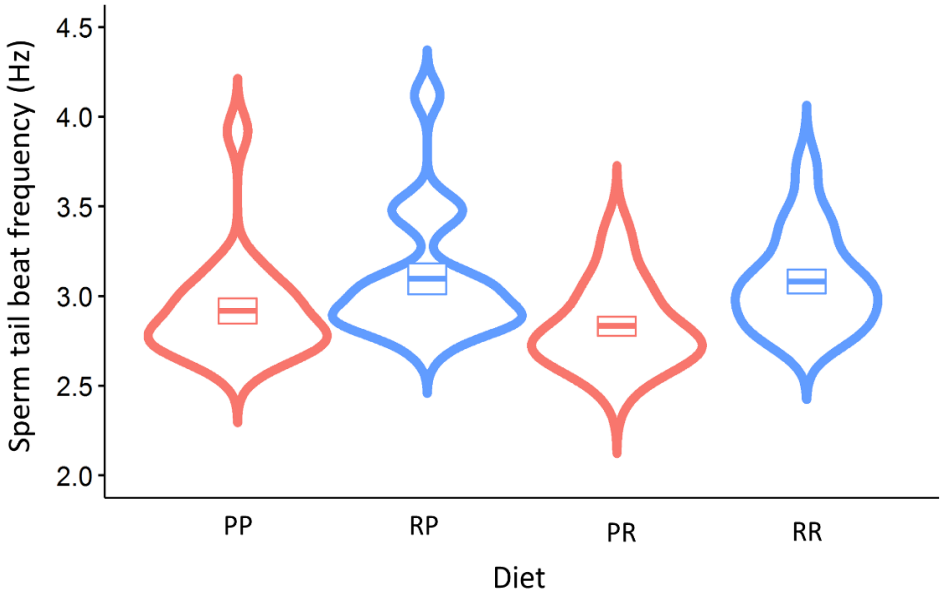


Figure 1. Violin plots showing dietary effects on sperm tail-beat frequency. The X axis shows all four diet combinations where P = nutrient poor diet, R = nutrient rich diet, the first letter corresponds to the developmental diet and the second letter corresponds to the adult diet (red plot = poor developmental diet, blue plot = rich developmental diet). Mean ± SE on inside of each plot.

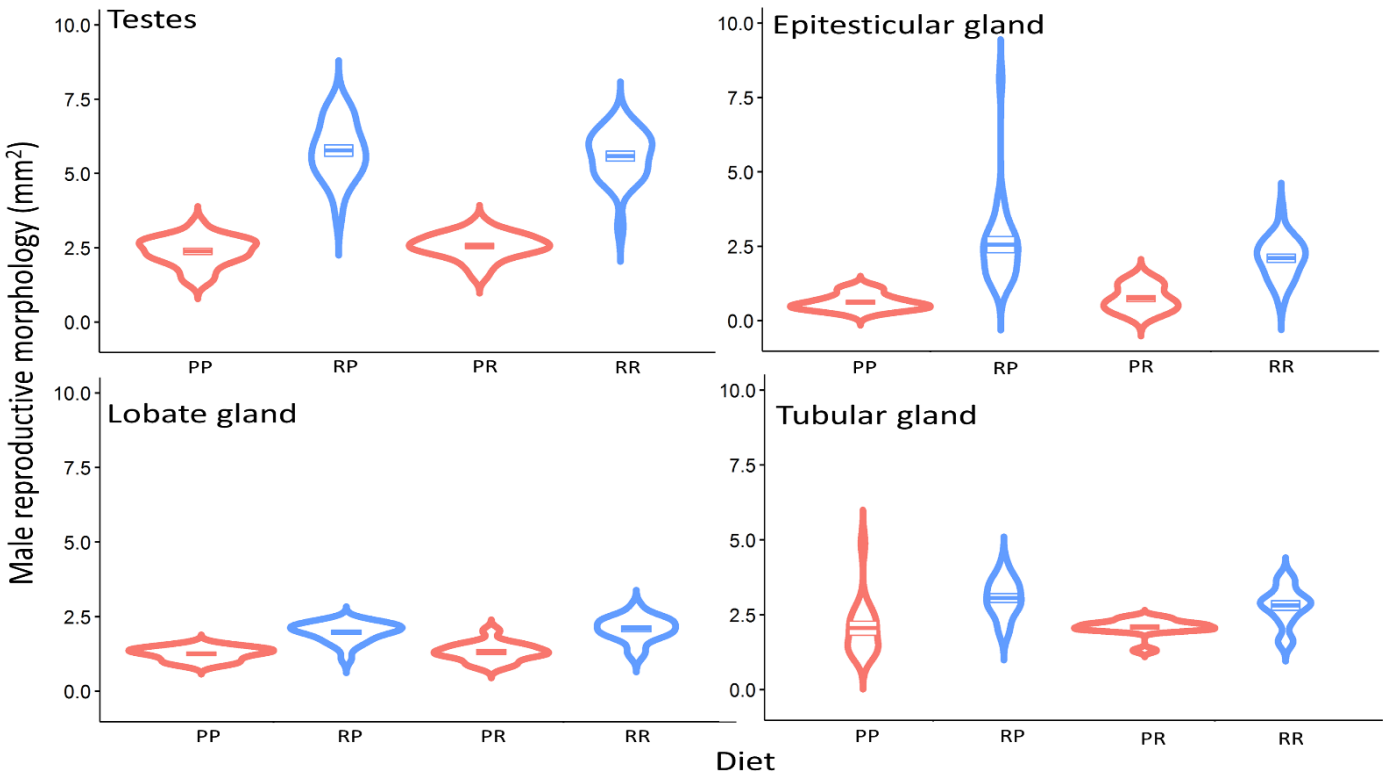


Figure 2. Violin plots showing dietary effects on male reproductive morphology. The X axis shows all four diet combinations where P = nutrient poor diet, R = nutrient rich diet, the first letter corresponds to the developmental diet and the second letter corresponds to the adult diet (red plot = poor developmental diet, blue plot = rich developmental diet). Mean ± SE on inside of each plot.

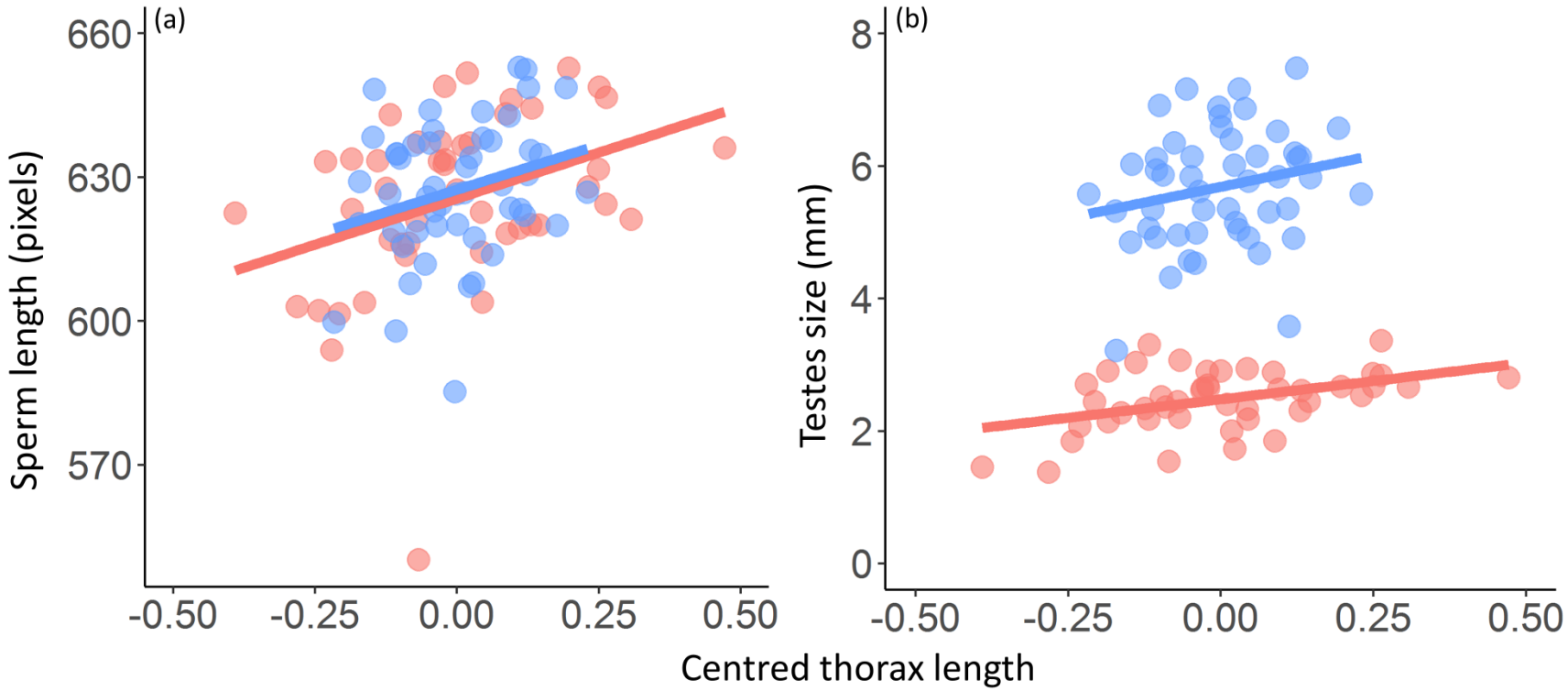


Figure 3. Relationship between thorax length (centred within developmental diet) and sperm length (a), and testes size (b), grouped by developmental diet treatment. Red dots and line = poor developmental diet, blue dots and line = rich developmental diet

Discussion

We found that developmental nutrient availability has a long-lasting and perhaps irreversible effect on post-copulatory reproductive investment in male neriid flies. Males reared on a nutrient-poor developmental diet were not only smaller, they also had smaller testes and accessory glands, and produced slower sperm, regardless of the quality of the diet they were fed as adults. Testes size and sperm length were positively correlated with thorax length centred within developmental diet treatment groups, suggesting that these traits are genetically correlated with body size (or potentially another environmental factor that is correlated with body size and was not controlled for in our study). Sperm size was also negatively associated with male age (in the narrow range of ages included in this study) yet was not affected by developmental or adult diet manipulations, indicating that sperm size does not respond plastically to dietary nutrients. Surprisingly, there was no effect of adult diet, nor an interactive effect of developmental and adult diet on any of the post-copulatory traits measured. This suggests that there is no compensatory or cumulative effect of adult diet and developmental diet, nor is there a benefit of environmental matching between developmental and adult diets in male neriid flies. Therefore, male post-copulatory traits appear to be most sensitive to developmental nutrients in *T. angusticollis*, with a nutrient-rich developmental diet providing a 'silver spoon' for post-copulatory trait expression.

Similar to our findings, male spiders (*Paratrechalea ornata*) that were subject to food deprivation early in life were unable to recover the quality of their saliva produced nuptial gifts when provided with increased food later in life (Macedo-Rego *et al.*, 2016). However, in rats, although sperm production was reduced by *in utero* dietary restriction, sperm production was recovered when males were provided with a normal protein diet after weaning (Melo *et al.*, 2014). These differences between species may reflect differences in ontogeny of post-copulatory traits. For example, in holometabolous insects like neriid flies, the larval (developmental) stage is the phase where nutrients are allocated to body growth and extra metabolites are stored for use in the adult stage (Boggs, 1981). Allocation to imaginal disks that develop into adult reproductive structures like testes and accessory glands also occurs during the

developmental stage. Nutrition during development may also shape epigenetic programming of imaginal disk tissues that develop into the male germ line, with life-long effects on gene-expression patterns in the testes (and potentially sperm, affecting sperm movement) and accessory glands. However, it is interesting that other holometabolous insects can compensate for a scarcity of developmental resources in other fitness enhancing traits (Mevi-Schütz & Erhardt, 2005; Müller & Müller, 2016), suggesting that regulation of post-copulatory trait expression can vary substantially between species.

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

Restricting protein in the adult diet increases lifespan of both male and female neriid flies by 65% but renders females completely infertile (Adler *et al.* 2013). Yet, despite these dramatic effects on lifespan and female reproduction, we found no effect of adult dietary protein restriction on male post-copulatory traits. Similarly, previous studies of *T. angusticollis* have only found subtle, context-dependent effects of adult diet on male reproduction (Adler *et al.* 2013; Macartney *et al.* 2017). Protein is a key nutrient for male reproduction in other species (Droney, 1998; Baker *et al.*, 2003; Costa *et al.*, 2012), and it is therefore surprising that it has little influence on male reproduction in *T. angusticollis.*

Male and female neriid flies require different quantities of developmental nutrients for optimal reproductive investment (Bonduriansky, Runagall-McNaull, & Crean, 2016), and this appears to equally apply to adult nutrient requirements. It is possible that restricting sugar in the adult diet may influence male reproductive traits. However, because *T. angusticollis* adults feed on tree sap, sugar is unlikely to be limiting in their natural diet, and substantial carbohydrate restriction causes rapid death (Adler et al. 2013).

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

Conclusions

Overall, there is no evidence that male testes and accessory gland size, sperm movement, or sperm length are affected by an interaction between developmental and adult nutrition in *Telostylinus angusticollis*, nor are male post-copulatory traits affected by adult nutrition. Instead, we show that sperm movement, testes size and accessory gland size are most sensitive to developmental nutrition and respond in a condition-dependent manner. A nutrient-rich developmental diet provides a 'silver spoon' for adult male post-copulatory trait expression, while males fed a nutrient-poor developmental diet are less able to invest in post-copulatory traits even if they encounter abundant nutrients as adults. This irreversible reduction in male post-copulatory trait expression may then reduce male fitness under sperm-competition

References

ADLER, M.I., CASSIDY, E.J., FRICKE, C. & BONDURIANSKY, R. (2013) The lifespan-reproduction trade-off under dietary restriction is sex-specific and context-dependent. *Experimental Gerontology* **48**, 539–548.

AMITIN, E.G. & PITNICK, S. (2007) Influence of developmental environment on male- and female-mediated sperm precedence in *Drosophila melanogaster*. *Journal of Evolutionary Biology* **20**, 381–391.

ANDERSSON, M. (1982) Sexual selection, natural selection, and quality advertisement. *Biological Journal of the Linnean Society* **17**, 375–393.

AVILA, F.W., SIROT, L.K., LAFLAMME, B.A., RUBINSTEIN, C.D. & WOLFNER, M.F. (2011) Insect seminal fluid proteins: identification and function. *Annual Review of Entomology* **56**, 21–40.

BAKER, R.H., DENNIFF, M., FUTERMAN, P., FOWLER, K., POMIANKOWSKI, A. & CHAPMAN, T. (2003) Accessory gland size influences time to sexual maturity and mating frequency in the stalk-eyed fly, *Cyrtodiopsis dalmanni*. *Behavioral Ecology* **14**, 607–611.

BARKER, D.J.P. (2004) The developmental origins of adult disease. *Journal of the American College of Nutrition* **23**, 588–595.

BARTLETT, M.J., STEEVES, T.E., GEMMELL, N.J. & ROSENGRAVE, P.C. (2017) Sperm competition risk drives rapid ejaculate adjustments mediated by seminal fluid. *eLife* **6**, 1–23.

BATES, D., MAECHLER, M., BOLKER, B. & WALKER, S. (2015) lme4: Linear mixed-effects models using Eigen and S4. *Journal of Statistical Software* **67**, 1–48.

BOGGS, C.L. (1981) Nutritional and life-history determinants of resource-allocation in holometabolous insects. *American Naturalist* **117**, 692–709.

BONDURIANSKY, R. (2007) The evolution of condition-dependent sexual dimorphism. *American Naturalist* **169**, 9–19.

BONDURIANSKY, R., RUNAGALL-MCNAULL, A. & CREAN, A.J. (2016) The nutritional geometry of parental effects: maternal and paternal macronutrient consumption and offspring phenotype in a neriid fly. *Functional Ecology* **30**, 1675–1686.

BOSCHETTO, C., GASPARINI, C. & PILASTRO, A. (2011) Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*).

Behavioral Ecology and Sociobiology **65**, 813–821.

CAMERON, E., DAY, T. & ROWE, L. (2007) Sperm competition and the evolution of ejaculate composition. *American Naturalist* **169**, 158–172.

CORNWALLIS, C.K. & BIRKHEAD, T.R. (2008) Plasticity in reproductive phenotypes reveals status-specific correlations between behavioural, morphological, and physiological sexual traits. *Evolution* **62**, 1149–1161.

COSTA, A.M., ANJOS-DUARTE, C.S., RORIZ, A.K.P., DIAS, V.S. & JOACHIM-BRAVO, I.S. (2012) Male diet and age influence to inhibit female remating in *Ceratitis capitata* (Diptera: Tephritidae). *Journal of Applied Entomology* **136**, 456–463.

CREAN, A.J., KOPPS, A.M. & BONDURIANSKY, R. (2014) Revisiting telegony: offspring inherit an acquired characteristic of their mother's previous mate. *Ecology Letters* **17**, 1545–1552.

CURTIS, S.K. & BENNER, D.B. (1991) Movement of spermatozoa of *Megaselia scalaris* (Diptera: Brachycera: Cyclorrhapha: Phoridae) in artificial and natural fluids. *Journal of Morphology* **210**, 85–99.

DÁVILA, F. & ARON, S. (2017) Protein restriction affects sperm number but not sperm viability in male ants. *Journal of Insect Physiology* **100**, 71–76.

DEWITT, T.J., SIH, A. & WILSON, D.S. (1998) Cost and limits of phenotypic plasticity. *Trends in Ecology & Evolution* **13**, 77–81.

DRONEY, D.C. (1998) The influence of the nutritional content of the adult male diet on testis mass, body condition and courtship vigour in a Hawaiian Drosophila. *Functional Ecology* **12**, 920–928.

DUPLOUY, A., WOESTMANN, L., GALLEGO ZAMORANO, J. & SAASTAMOINEN, M. (2017) Impact of male condition on his spermatophore and consequences for female reproductive performance in the Glanville fritillary butterfly. *Insect Science* **25**, 1–13.

EVANS, J.P., RAHMAN, M.M. & GASPARINI, C. (2015) Genotype-by-environment interactions underlie the expression of pre- and post-copulatory sexually selected traits in guppies. *Journal of Evolutionary Biology* **28**, 959–972.

FRICKE, C., ADLER, M.I., BROOKS, R.C. & BONDURIANSKY, R. (2015) The complexity of male reproductive success: effects of nutrition, morphology, and experience. *Behavioral Ecology* **26**, 617–624.

GHEORGHE, C., GOYAL, R., MITTAL, A. & LONGO, L. (2010) Gene expression in the placenta: Maternal stress and epigenetic responses. *International Journal of Developmental Biology* **54**, 507–523.

HELLRIEGEL, B. & BLANCKENHORN, W.U. (2002) Environmental influences on the gametic investment of yellow dung fly males. *Evolutionary Ecology* **16**, 505–522.

HOOPER, A.K., SPAGOPOULOU, F., WYLDE, Z., MAKLAKOV, A.A. & BONDURIANSKY, R. (2017) Ontogenetic timing as a condition-dependent life history trait: high-condition males develop quickly, peak early and age fast. *Evolution* **71**, 671–685.

KAHRL, A.F. & COX, R.M. (2015) Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behavioral Ecology* **26**, 1502–1511.

KALDUN, B. & OTTI, O. (2016) Condition-dependent ejaculate production affects male mating behavior in the common bedbug *Cimex lectularius*. *Ecology and Evolution* **6**, 2548–2558.

KUZNETSOVA, A., BROCKHOFF, P. & RUNE, H. (2017) lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software* **82**, 1–26.

LINKLATER, J.R., WERTHEIM, B., WIGBY, S. & CHAPMAN, T. (2007) Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. *Evolution* **61**, 2027–2034.

LÜPOLD, S. & PITNICK, S. (2018) Sperm form and function: what do we know about the role of sexual selection? *Reproduction* **155**, R229–R243.

MACARTNEY, E.L., CREAN, A.J. & BONDURIANSKY, R. (2017) Adult dietary protein has age- and context-dependent effects on male post-copulatory performance. *Journal of Evolutionary Biology* **38**, 42–49.

MACEDO-REGO, R.C., COSTA-SCHMIDT, L.E., SANTOS, E.S.A. & MACHADO, G. (2016) Negative effects of prolonged dietary restriction on male mating effort: nuptial gifts as honest indicators of long-term male condition. *Scientific Reports* **6**, 1–8.

MEHLIS, M., RICK, I.P. & BAKKER, T.C.M. (2015) Dynamic resource allocation between pre- and postcopulatory episodes of sexual selection determines competitive fertilization success. *Proceedings of the Royal Society B: Biological Sciences* **282**, 1–9.

MELO, M.C., ALMEIDA, F.R.C.L., CALDEIRA-BRANT, A.L., PARREIRA, G.G. & CHIARINI-GARCIA, H. (2014) Spermatogenesis recovery in protein-restricted rats subjected to a normal protein diet after weaning. *Reproduction, Fertility and Development* **26**, 787–796.

MEVI-SCHÜTZ, J. & ERHARDT, A. (2005) Amino acids in nectar enhance butterfly fecundity: a long-awaited link. *The American Naturalist* **165**, 411–419.

MONAGHAN, P. (2008) Early growth conditions, phenotypic development and environmental change. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363**, 1635–1645.

MÜLLER, T. & MÜLLER, C. (2016) Adult beetles compensate for poor larval food conditions. *Journal of Insect Physiology* **88**, 24–32.

NICOVICH, P.R., MACARTNEY, E.L., WHAN, R.M. & CREAN, A.J. (2015) Measuring sperm movement within the female reproductive tract using Fourier Analysis. *Microscopy and Microanalysis* **21**, 256–263.

NUR, N. & HASSON, O. (1984) Phenotypic plasticity and the handicap principle. *Journal of Theoretical Biology* **110**, 275–297.

PARKER, G.A. (1990) Sperm competition games: raffles and roles. *Proceedings of the Royal Society B: Biological Sciences* **242**, 120–126.

PARKER, G.A. & PIZZARI, T. (2010) Sperm competition and ejaculate economics. *Biological Reviews* **85**, 897–934.

PERRY, J.C. & ROWE, L. (2010) Condition-dependent ejaculate size and composition in a ladybird beetle. *Proceedings of the Royal Society B: Biological Sciences* **277**, 3639–3647.

PERRY, J.C., SIROT, L. & WIGBY, S. (2013) The seminal symphony: how to compose an ejaculate. *Trends in Ecology & Evolution* **28**, 414–422.

QVARNSTROM, A. & PRICE, T.D. (2001) Maternal effects, paternal effects and sexual selection. *Trends in Ecology & Evolution* **16**, 95–100.

RAHMAN, M.M., KELLEY, J.L. & EVANS, J.P. (2013) Condition-dependent expression of pre- and postcopulatory sexual traits in guppies. *Ecology and Evolution* **3**, 2197–2213.

RASBAND, W.S. (2015) ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA. *http://imagej.nih.gov/ij/*.

REINHARDT, K., DOBLER, R. & ABBOTT, J. (2015) An ecology of sperm: sperm

diversification by natural selection. *Annual Review of Ecology, Evolution, and Systematics* **46**, 435–459.

ROWE, L. & HOULE, D. (1996) The lek paradox and the capture of genetic variance by condition dependent traits. *Proceedings of the Royal Society B: Biological Sciences* **263**, 1415–1421.

RUNAGALL-MCNAULL, A., BONDURIANSKY, R. & CREAN, A.J. (2015) Dietary protein and lifespan across the metamorphic boundary: protein-restricted larvae develop into short-lived adults. *Scientific Reports* **5**, 1–7.

SCHAALJE, G.B., MCBRIDE, J.B. & FELLINGHAM, G.W. (2002) Adequacy of approximations to distributions of test statistics in complex mixed linear models. *Journal of Agricultural, Biological, and Environmental Statistics* **7**, 512–524.

SCHÄRER, L., LADURNER, P. & RIEGER, R.M. (2004) Bigger testes do work more: experimental evidence that testis size reflects testicular cell proliferation activity in the marine invertebrate, the free-living flatworm Macrostomum sp. *Behavioral Ecology and Sociobiology* **56**, 420–425.

SENTINELLA, A., CREAN, A. & BONDURIANSKY, R. (2013) Dietary protein mediates a trade-off between larval survival and the development of male secondary sexual traits. *Functional Ecology* **27**, 1134–1144.

SIMMONS, L.W., TOMKINS, J.L. & HUNT, J. (1999) Sperm competition games played by dimorphic male beetles. *Proceedings of the Royal Society B: Biological Sciences* **266**, 145–150.

TOMÁŠEK, O., ALBRECHTOVÁ, J., NĚMCOVÁ, M., OPATOVÁ, P. & ALBRECHT, T. (2017) Trade-off between carotenoid-based sexual ornamentation and sperm resistance to oxidative challenge. *Proceedings of the Royal Society of London B: Biological Sciences* **284**, 1–9.

VEGA-TREJO, R., JENNIONS, M.D. & HEAD, M.L. (2016) Are sexually selected traits affected by a poor environment early in life? *BMC Evolutionary Biology* **16**, 1–12.

VELLNOW, N., MARIE-ORLEACH, L., ZADESENETS, K.S. & SCHÄRER, L. (2018) Bigger testes increase paternity in a simultaneous hermaphrodite, independently of the sperm competition level. *Journal of Evolutionary Biology* **31**, 180–196.

VERMEULEN, A., ENGELS, S. & SAUER, K.P. (2008) Maintenance of variance in

sperm transfer rates in a scorpionfly: food availability, genetic basis, and heritability. *Behavioral Ecology and Sociobiology* **63**, 77–83.

WERNER, M., GACK, C., SPECK, T. & PESCHKE, K. (2007) Queue up, please! Spermathecal filling in the rove beetle Drusilla canaliculata (Coleoptera, Staphylinidae). *Naturwissenschaften* **94**, 837–841.

WIGBY, S., PERRY, J.C., KIM, Y.H. & SIROT, L.K. (2016) Developmental environment mediates male seminal protein investment in Drosophila melanogaster. *Functional Ecology* **30**, 410–419.

WILLIAMS, P., DAY, T. & CAMERON, E. (2005) The evolution of sperm-allocation strategies and the degree of sperm competition. *Evolution* **59**, 492–499.

Supplementary material

Sperm movement within the female reproductive tract methods

Not all males transferred sperm at mating, but the probability of sperm transfer was not influenced by treatment (proportion of males that failed to transfer sperm: RR = 8/26; RP= 10/27; PR = 7/26; PP = 7/25; LRT additive versus null model $\chi^2 = 0.688$ p = 0.709).

Videos of sperm movement were captured under darkfield illumination using a Zeiss AxioCam HSc high speed camera mounted on a Zeiss AxioScope A1 compound microscope, and time-lapse image stacks recorded using Axiovision software (Carl Zeiss Microscopy, Germany). Sperm movement was recorded for 60s, with an average frame rate of 60 frames/second. The average time from mating to recording was 4.5 ± 1.3 minutes (mean ± SD). Fourier analysis was performed on intensity versus time traces extracted on a pixel-by-pixel basis from the time-stamped image stacks using a custom-written GUI in MATLAB (see Nicovich *et al.,* 2015 for details). Time traces were high-pass filtered at 2.5Hz to suppress slow-motion movements such as muscle contractions of the reproductive tract. The frequency position of the maximum of the Fourier transform of the filtered trace was taken as the average beat frequency at that pixel, and a map of beat frequency with respect to position was generated.

Saved MATLAB data files of the pixel-based average beat frequency maps were loaded into a second MATLAB-based GUI program that calculated descriptive statistics of pixels within user-defined regions-of-interest (ROI). ROIs were selected by tracing around the internal perimeter of spermathecae and straight sections of sperm ducts, leaving a slight gap from the edge to avoid wall effects. Estimates of median tail beat frequency were calculated from each separate duct and spermathecae visible within each video. The ROI with the maximum median tail beat frequency was used as a measure of sperm movement within the female reproductive tract for each individual male.

Male reproductive morphology measurements

We quantified the size of three accessory glands. These were the 'tubular glands' (Fig. S1(a)) that arise from the base of the testes, as well as two other structures that appear to be glandular: the 'lobate glands' (Fig. S1(a)) and the 'epitesticular glands' (Fig. S1(b)). The location of the three accessory glands relative to the testes and sperm duct is shown in Fig S1(c).

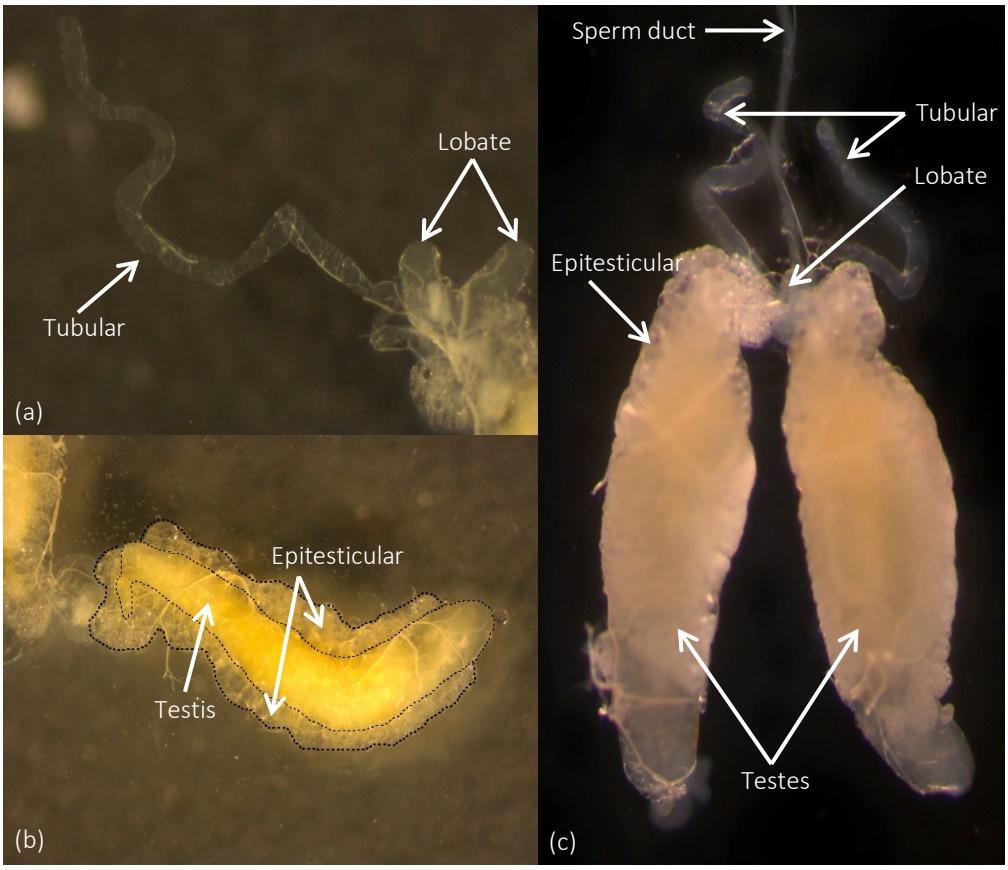


Figure S1. Putative accessory glands and testis (a, b) and internal reproductive structures of *Telostylinus angusticollis* (c).

Likelihood-ratio-tests

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

	In vivo sperm motility	Testes size	Sperm length	Epitesticular accessory gland	Lobate accessory gland	Tubular accessory gland
χ^2	0.577	0.296	**15.769**	0.792	0.400	1.001
p value	0.448	0.586	**<0.001**	0.373	0.527	0.3172

Means and standard errors for the effect of diet on thorax and post-copulatory traits

Table S2. Means and standard errors for each response variable in each combination of developmental and adult diet. First letter corresponds to developmental diet and second letter corresponds to adult diet (P=poor, R=rich).

Diet	Thorax size (mm)	Sperm tail beat frequency (Hz)	Testes size (mm^2)	Sperm length (pixels)	Epitesticular accessory gland (mm^2)	Lobate accessory gland (mm^2)	Tubular accessory gland (mm^2)
PP	1.970 ± 0.040	2.900 ± 0.045	2.507 ± 0.095	636.531 ± 2.471	0.622 ± 0.062	1.234 ± 0.049	1.915 ± 0.121
PR	1.878 ± 0.031	2.842 ± 0.080	2.441± 0.095	615.379 ± 3.454	0.747 ± 0.098	1.355 ± 0.086	2.233 ± 0.209
RP	2.905 ± 0.018	3.193 ± 0.094	5.862 ± 0.163	630.432 ± 3.511	2.332 ± 0.155	2.041 ± 0.084	2.856 ± 0.139
RR	2.905 ± 0.021	3.089 ± 0.049	5.486 ± 0.184	624.383 ± 2.625	2.101 ± 0.132	2.027 ± 0.373	3.053 ± 0.170

Chapter Four

EPIGENETIC PATERNAL EFFECTS AS COSTLY, CONDITION-DEPENDENT TRAITS

Erin L Macartney, Angela J Crean, Russell Bonduriansky

Heredity (2018). ***121****, 248–256*

ELM conceived and wrote the manuscript. AJC and RB provided valuable suggestions throughout the writing of this manuscript.

Abstract

It is now recognized that post-copulatory traits such as sperm and ejaculate production can impose metabolic costs, and such traits are therefore expected to exhibit condition-dependent expression whereby low condition individuals experience a greater marginal cost of investment compared to high condition individuals. Ejaculates are especially costly in species where males invest in offspring quality through nutrient-rich spermatophores or other seminal nuptial gifts. However, recent evidence shows that, in species where males do not provision females or offspring, males can still influence offspring development through paternal effects mediated by epigenetic factors such as non-coding RNAs, DNA methylation and chromatin structure. Because such epigenetic paternal effects do not involve the transfer of substantial quantities of resources such as nutrients, the costs of conferring such effects have not been considered. Here, we argue that if selection favours paternal investment in offspring quality through epigenetic factors, then the epigenetic machinery required to bring about such effects may also be expected to evolve strongly condition-dependent expression. We outline indirect evidence suggesting that epigenetic paternal effects could impose substantial metabolic costs, consider the conditions under which selection may act on such effects, and suggest ways to test for differential costs and condition-dependence of these effects. Incorporating epigenetic paternal effects into condition-dependent life history theory will further our understanding of the heritability of fitness and the evolution of paternal investment strategies.

Epigenetics and life history

Recent evidence shows that males across many taxa, including nematodes, insects, fish and mammals, can influence offspring development and quality through epigenetic factors transferred in the sperm and/or semen (reviewed in Crean & Bonduriansky, 2014; Rando, 2016; Wang *et al.*, 2017). These epigenetic factors can include small noncoding RNAs (nc-RNAs), DNA methylation, and chromatin structure, and all these factors can alter gene-expression in developing embryos (e.g., Milekic *et al.*, 2015; Grandjean *et al.*, 2015; Skinner, 2016; Klosin *et al.*, 2017).

Modifications of the methylation pattern or differences in chromatin structure of the paternal haploid genome in sperm can in some cases be retained throughout offspring embryonic development or even into adulthood, affecting important aspects of offspring phenotype and fitness (Guerrero-Bosagna *et al.*, 2010; Manikkam *et al.*, 2012; Vassoler *et al.*, 2012; Kelly, 2014; Klosin *et al.*, 2017), and noncoding RNAs (such as miRNAs or tsRNAs) can be transferred to the zygote in the sperm and/or seminal fluid and can also alter gene expression in the offspring (Gapp *et al.*, 2014; Stoeckius *et al.*, 2014; Rodgers *et al.*, 2015; Marré *et al.*, 2016). Moreover, changes in multiple types of epigenetic factors often appear to be involved in paternal effects on offspring fitness. For example, high fat diets in mice can alter the expression of miRNAs, methylation patterns, and chromatin structure in the paternal germline (Fullston *et al.*, 2013; Duale *et al.*, 2014; Barbosa *et al.*, 2016), and these can then up- or down- regulate gene expression in offspring (affecting genes involved in metabolism, insulin secretion and glucose tolerance, and embryo development) and cause significant reductions in offspring health (e.g., Ng *et al.*, 2010; Binder *et al.*, 2012a, b; Fullston *et al.*, 2013; Mitchell *et al.*, 2017). While these well-characterised paternal effects act to reduce offspring fitness, there are many examples of non-genetic paternal effects that have the potential to increase offspring fitness by enhancing offspring survival and/or reproductive success (e.g., Bonduriansky & Head, 2007; Carone *et al.*, 2010; Crean *et al.*, 2013; Delcurto *et al.*, 2013; Jensen *et al.*, 2014; Evans *et al.*, 2017). Paternal effects with both negative and positive effects on offspring fitness can be mediated by similar epigenetic mechanisms. Yet, despite their potential

importance, there has been little to no incorporation of epigenetic paternal effects into life history theory.

A central idea in life history theory is that persistent directional selection on fitness-enhancing traits can lead to trait exaggeration and thereby drive up the metabolic cost required to produce the trait. Therefore, the amount of metabolic resources available to an individual (i.e., its condition) is expected to determine the expression of such a costly trait, resulting in condition-dependent trait expression (Andersson, 1982; Nur and Hasson, 1984; Grafen, 1990; Iwasa *et al.*, 1991; Kotiaho, 2001; Cotton *et al.*, 2004). Individuals that have fewer metabolic resources (i.e., are in low condition) are less able to invest in costly traits compared to individuals that have more metabolic resources (i.e., are in high condition)—that is, low condition individuals experience a higher marginal cost of trait expression. This theory has been extensively applied to secondary sexual traits such as displays and weaponry (Moller & Delope, 1994; Mappes *et al.*, 1996; Kotiaho, 2000; Judge *et al.*, 2008) and, more recently, to post-copulatory traits such as sperm quality and quantity (reviewed in Fitzpatrick & Lüpold 2014; Lüpold et al. 2016; Godwin et al. 2017) and ejaculate size and composition (Gwynne 1993; Eberhard & Cordero, 1995; Linklater *et al.*, 2007; Perry & Tse, 2013; Friesen *et al.*, 2015; Bretman *et al.*, 2016; Wigby *et al.*, 2016). All these traits are important for male reproductive success and can therefore be exaggerated by selection.

Selection can also favour paternal investment in offspring quality (Maynard Smith, 1977; Clutton-Brock, 1991; Sheldon, 2002; Requena and Alonzo, 2017). The costs and condition-depedence of paternal investment have been examined in species where males directly provision their offspring through paternal care (reviewed in Clutton-Brock 1991; Badyaev & Hill 2002; Kelly & Alonzo 2009), or confer nutrient-rich spermatophores or other types of glandular nuptial gifts to females (Gwynne and Simmons, 1990; Michaud *et al.*, 2013; Mirhosseini *et al.*, 2014). Such seminal provisioning is typically associated with the production of very large ejaculates that are expected to require substantial investment of resources and to impose substantial metabolic costs.

However, such obvious forms of paternal investment are lacking in most species (Eberhard 1997).

More recently, it has been recognised that paternal investment may extend beyond parental care and nutrient provisioning, with calls to generalize the definition of parental investment beyond a 'nutrition-centric' view to include any investment in an offspring that reduces the parent's ability to invest in future offspring (Royle *et al.*, 2012; also see Trivers, 1972). If the molecular mechanisms that mediate the transmission of epigenetic factors from fathers to their offspring are costly to build, maintain and deploy, then epigenetic paternal effects are encompassed by this definition of paternal investment. Below, we argue that epigenetic paternal effects that enhance offspring fitness are indeed likely to be costly, and that such effects should be incorporated into life-history theory as condition-dependent paternal investment traits. In addition to behavioural and nutritional provisioning, males may increase offspring survivial and/or reproductive success through investment in molecular processes that shape the epigenome in the paternal germ-line and determine the nature of the epigenetic factors transferred to offspring via the sperm and seminal fluid. This can then provide variation for selection to act on, with the fitter offspring surviving to confer the ability to invest in offspring through such epigenetic molecular mechanisms. Selection for enhanced offspring fitness through epigenetic inheritance may then further drive up the metabolic cost of molecular investment, resulting in strongly condition-dependent investment, like that observed in other fitness-enhancing traits (Moller and Delope, 1994; Rowe and Houle, 1996; Kotiaho, 2000; Perry and Rowe, 2010).

Obviously, epigenetic factors transmitted through the germ-line can also mediate maternal effects (reviewed in Aiken & Ozanne, 2016). However, because maternal effects can occur via a wide range of mechanisms, such as the egg cytoplasm, the intrauterine environment, or post-partum provisioning (Champagne, 2008), the role of germ-line epigenetic factors can be difficult to establish and such factors are unlikely to constitute a major component of total maternal investment. By contrast, in species where males do not provide

parental care or nutritional resources, epigenetic paternal effects are likely to comprise a large share of total paternal investment. We therefore focus our discussion on paternal effects mediated by epigenetic factors.

Is epigenetic machinery costly to build and maintain?

Several lines of evidence suggest that the cost of maintaining a 'good' epigenetic profile could be substantial, and individuals that are unable to invest in maintaining a good epigenome are likely to produce lower quality offspring. Firstly, changes in chromatin structure, RNA synthesis, DNA methylation and some de-methylation require energetic and material investment in tightly regulated molecular processes. Such processes include histone acetylation, RNA synthesis, and the expression and deployment of DNA methyltransferases (DNMTs) and methyl-CpG-binding domain (MBD) proteins. All these processes require ATP to provide energy to build and deploy (Gaal et al., 1997; Amiott and Jaehning, 2006; Wellen et al., 2009; Bhutani et al., 2011; Horvath, 2013). Therefore, individuals that do not have substantial metabolic reserves may be less able to invest in these metabolic pathways.

The most extensively studied of these epigenetic factors is DNA methylation. In mammals and plants, it has been shown that newly synthesised DNA lacks methylation until maintenance methyltransferases (DNMT1) restore methylation patterns through some type of memory mechanism (Okano *et al.*, 1999; Saze *et al.*, 2003; Kato *et al.*, 2007). Horvath (2013) proposed that a substantial amount of energy is needed to maintain epigenetic stability during the stressful period of development when the rate of cell division is high. This may explain why we see such pronounced effects of males' developmental environment on their subsequent capacity to influence the development of their offspring (Bonduriansky & Head, 2007; Burdge *et al.*, 2007; Kaati *et al.*, 2007; Bonduriansky *et al.*, 2016). Horvath (2013) also suggests that constant energy expenditure may be required to maintain epigenetic stability throughout adult life, given that DNMTs need to be deployed to maintain existing methylation patterns. Any perturbations such as stress or exposure to toxins may therefore lead to epigenetic dysregulation.

If both the establishment and maintenance of epigenetic machinery are costly, then environmental conditions both during development and during adult life may be expected to affect the epigenome. Such costs may be expected to apply to the maintenance of the epigenome in the soma as well as the germ-line, where epigenetic changes resulting from environmental perturbations can be transmitted to offspring (Lambrot *et al.*, 2013; Guerrero-Bosagna and Skinner, 2014; Kitamura *et al.*, 2015). This could explain why both juvenile and adult environments are sometimes found to influence paternal effects on offspring fitness (Ducatez *et al.*, 2012; Adler & Bonduriansky, 2013; Braun & Champagne, 2014; Fricke *et al.*, 2015; Macartney *et al.*, 2017). However, some paternal effects could be programmed during a specific ontogenetic phase. For example, if the epigenetic machinery involved in such effects is built during embryonic development, then the nutrient abundance or stress experienced by males during a specific sensitive phase of development could largely determine the paternal effects that the males will confer as adults if environmental perturbations also disrupt epigenetic regulation of the germ line (e.g., Bonduriansky and Head, 2007; Kaati *et al.*, 2007; Macartney *et al.*, 2017).

Hypomethylation and (to a lesser extent) hypermethylation of some sites occur with age throughout the mammalian genome—a process known as the 'epigenetic clock' (Bellizzi *et al.*, 2012; Horvath, 2013; Marttila *et al.*, 2015; Milekic *et al.*, 2015; Breitling *et al.*, 2016). Changes in chromatin structure and RNA transcriptional dysfunction have also been shown to increase with age (reviwed in Ashapkin *et al.*, 2017), and several studies have shown that these epigenetic changes to DNA methylation, chromatin structure and RNA synthesis can be accelerated by stress and toxins (Dick *et al.*, 2014; Duale *et al.*, 2014; Horvath *et al.*, 2014; Beach *et al.*, 2015; Boks *et al.*, 2015; Gao *et al.*, 2016). These changes in epigenetic regulation probably reflect negative effects of age and stress on the epigenetic maintenance system (Bellizzi *et al.*, 2012; Horvath, 2013; Breitling *et al.*, 2016). Such epigenetic dysregulation has been demonstrated to occur in the germ-line as well as the soma (e.g., Lambrot *et al.*, 2013; Duale *et al.*, 2014; Milekic *et al.*, 2015; Rodgers *et al.*, 2015), suggesting that some age- and stress-induced epigenetic changes can be transmitted to offspring through transgenerational epigenetic inheritance (Miller *et al.*, 2010;

Danchin *et al.*, 2011; Seong *et al.*, 2011; Jenkins and Carrell, 2012; Soubry, 2015). Just as individuals that are in high condition can prevent or repair genetic mutations better than individuals in low condition (Agrawal & Wang, 2008; Skinner *et al.*, 2015; Skinner, 2016), males in high condition may be better able to protect or repair the epigenome of their soma and germ-line from age- and stress-induced dysregulation, and thereby produce offspring of higher quality.

In addition to the energetic costs of investing in protection and repair of the epigenome, the ability to synthesize epigenetic factors can be limited by access to certain biochemicals. For example, methylation requires methyl groups, which are derived from dietary methionine—an amino acid that can be limited by the availability of certain foods (Grandison *et al.*, 2009), and dietary glucose appears to play an important role in histone acetylation which influences chromatin structure (Burdge & Lillycrop 2010). Therefore, access to specific dietary nutrients as well as metabolic energy can influence and limit the expression of epigenetic factors, and thereby affect the maintenance and repair of the epigenome.

Selection on epigenetic paternal effects

As with investment in other forms of paternal provisioning, selection for males to invest in offspring quality through epigenetic paternal effects will only occur under certain conditions (Kokko, 1999; Kokko & Jennions, 2008; West & Capellini, 2016; Requena & Alonzo, 2017). Selection may occur directly through female mate choice, if females discriminate among males based on an honest signal of paternal epigenetic investment. While male sexual signals typically exhibit condition-dependent expression, some components of the male phenotype may specifically reveal male epigenetic quality and such signals should be investigated in the future. Females could evolve preferences based on these signals, and such signaler-receiver coevolution could be prevalent in non-resource-based systems where conventional forms of paternal investment are lacking (Crean et al. 2016).

Selection may also occur indirectly, if the epigenetic paternal effect enhances offspring fitness, such that offspring are more likely to inherit and pass on genetic alleles that cause the development of the required epigenetic machinery. The fitness gains from any form of paternal investment will depend on paternity certainty, which reflects the likelihood of female re-mating and the risk of cuckoldry (i.e., the use of resources provided by one male to enhance the quality of another male's offspring) (e.g., Wickler, 1985; Gwynne, 1988). When the risk of cuckoldry is high, selection may instead favour males that invest in traits that enhance mating success. However, if paternal investment is conferred through factors transferred within the sperm and associated with paternal DNA (for example, via DNA methylation, chromatin structure, and sperm-borne ncRNAs), the risk of cuckoldry will be negligible or absent as the epigenetic factors are lied directly to fertilisaiton. Therefore, paternal investment mediated by sperm-borne epigenetic factors may be more likely to evolve than other mechanisms of paternal investment (Bonduriansky & Day 2013). Paternal investment though epigenetic factors may therefore be taxonomically widespread.

In particular, males of some species can confer their condition to offspring through epigenetic factors (Bonduriansky and Crean, 2017), with high condition males producing better quality offspring relative to low condition males (e.g., Bonduriansky & Head, 2007; Carone *et al.*, 2010; Delcurto *et al.*, 2013; Evans *et al.*, 2017; Zajitschek *et al.*, 2017). For example, such condition transfer effects have recently been reported in the guppy *Poecilia reticulata*, where epigenetic factors attached to the sperm of fathers reared on a high quantity diet produced larger offspring and probably enhanced juvenile survival (Evans *et al.*, 2017). Such effects have also been demonstrated in the neriid fly, *Telostylinus angusticollis,* where fathers reared on a nutrient rich diet produce larger offspring (Bonduriansky and Head, 2007), likely through epigenetic factors (Crean *et al.*, 2014).

While larger body size may be advantageous across a wide range of environments, males of some species may also anticipate the environment that their offspring are likely to experience and produce offspring that are better

suited to that environment ('anticipatory effects') (Marshall & Uller, 2007**;** Burgess & Marshall, 2014)**.** For example, Crean *et al.* (2013) and Jensen *et al.*, (2014) demonstrated anticipatory effects of male environment in a broadcast spawning ascidian (*Styela plicata*) and marine tubeworm (*Hydroides diramphus*) respectively, most likely through epigenetic changes to the sperm. Both condition-transfer and anticipatory effects can enhance offspring fitness, providing an indirect benefit to the father and generating positive selection on the cellular and physiological mechanisms involved in the paternal effect. Both condition-transfer and anticipatory effects are also likely to be costly for males, requiring the synthesis, maintenance and deployment of epigenetic factors that alter offspring development.

Conversely, epigenetic paternal effects can be detrimental. As mentioned previously, stressed or senescent individuals can undergo epigenetic dysregulation (Jirtle and Skinner, 2007; Horvath, 2013), and transmit some of these epigenetic changes to their offspring (e.g., Rassoulzadegan *et al.*, 2006; Manikkam *et al.*, 2012; Weyrich *et al.*, 2016). This can then result in offspring with decreased health and increased susceptibility to disease (Miller *et al.*, 2010; Danchin *et al.*, 2011; Seong *et al.*, 2011; Jenkins and Carrell, 2012; Rando, 2012; Soubry, 2015). Marshall & Uller (2007) suggest that such 'transmissive' effects occur due to physiological constraints on the expression of reproductive traits. Therefore, selection should favour males that are able to overcome such physiological constraints by investing more metabolic resources in maintaining a healthy germ-line epigenome to produce healthier offspring. High condition individuals possess more metabolic resources and may therefore be better able to prevent transmissive effects by investing in costly molecular mechanisms that protect or repair the epigenome.

Predictions and empirical tests

There is currently a dearth of empirical and theoretical work directly exploring the costs and condition-dependence of investment in epigenetic factors, including the molecular machinery involved in nongenetic paternal effects. Two important questions that require empirical research and that are key to furthering our understanding of the evolution and ecology of paternal effects

mediated by epigenetic factors are: 1) Under what conditions does selection favour male ability to influence offspring quality through transmission of beneficial epigenetic factors via the germ-line and/or through suppression of detrimental epigenetic effects?; and 2) does investment in epigenetic paternal effects result in life-history trade-offs similar to the trade-offs that limit investment in other costly reproductive traits?

To address question (1), it is necessary to determine whether males that confer positive epigenetic effects through their germ-line have higher fitness than males that do not confer such effects, and whether variation in the ability to confer such paternal effects is heritable. If both conditions are met, then the ability to confer such epigenetic effects to offspring may be expected to evolve. It would also be interesting to determine whether females preferentially mate with males that produce better quality offspring via such epigenetic effects, given that female preferences could contribute to selection on males to confer such effects (Bonduriansky & Day 2013; Bonilla et al. 2016; Head *et al.*, 2016). For example, a model by Bonduriansky and Day (2013) showed that paternal condition-transfer effects in species where males do not provide conventional forms of paternal provisioning could drive the evolution of costly female mate choice. Such female preferences could drive increased male investment in offspring quality. Importantly, given the potential for epigenetic paternal effects, the evolution of paternal investment can occur in species where opportunity for conventional forms of paternal investment (such as paternal care or nutrient-laden nuptial gifts) is lacking. For example, such effects can evolve in species such as guppies (Evans *et al.*, 2017) and neriid flies (Bonduriansky and Head, 2007) or in broadcast spawning species (Crean *et al.,* 2013; Jensen *et al.*, 2014), where males transfer small ejaculates and do not interact with their offspring.

Regarding question (2), if investment in offspring quality through epigenetic factors carries a substantial metabolic cost, then such investment may be predicted to respond to variation in the availability of metabolic resources, and to trade off against investment in other costly fitness components, as predicted by life history theory (Stearns, 1989; Zera and Harshman, 2001; Roff and Fairbairn, 2007). For example, we might expect to see a decline in offspring

quality with increased mating (or an increase in other costly life-history traits that may trade off with investment in offspring) due to a reduction in the ability to maintain the synthesis and/or maintenance of epigenetic factors in the germ-line. We may also expect to see a steeper decline in low condition individuals compared to high condition individuals if investing in these factors is condition-dependent. This has been demonstrated in males that produce spermatophores: in such species, depletion of male stores through repeated mating can reduce spermatophore size (Rutowski, 1979; Marcotte *et al.*, 2007; Michaud *et al.*, 2013) and alter offspring development (Michaud et al. 2013; Mirhosseini et al. 2014). However, such reductions in offspring quality have not yet been demonstrated in species where paternal effects on offspring performance are mediated by epigenetic factors. We may also expect to see a trade-off with other life-history traits, such as somatic maintenance and lifespan, as observed in males that transfer costly spermatophores (Mishra and Omkar, 2006; Perry and Tse, 2013). However, in order to directly test for costs of investment in epigenetic factors, and determine whether investment is condition-dependent (i.e., whether investment involves differential marginal costs to high condition vs. low condition males), male condition and the expression of the epigenetic factors that mediate paternal effects will need to be manipulated (as suggested by Kotiaho (2001) in relation to the costs and condition-dependence of secondary sexual traits).

Epigenetic factors could be manipulated by creating 'knock-out' lines for particular RNAs, administering oligo-nucleotides or synthesized RNAs, or by using CRISPR-Cas based tools (e.g., Vasudevan *et al.*, 2007; McDonald et al. 2016; Abudayyeh et al. 2016). These approaches may allow researchers to experimentally up- or down-regulate the expression of specific epigenetic factors involved in paternal effects or epigenetic regulation systems such as DNMT1s that maintain epigenetic integrity, and then measure how males of different condition respond to changes in such factors. For example, if the expression of an epigenetic factor is up-regulated, we may expect males of low condition to suffer a steeper decline in other life-history traits because of the higher marginal costs of investment in the epigenetic machinery. We may also detect an exaggerated decline in other life-history traits if the epigenetic

machinery is up-regulated in older individuals, since older individuals may suffer a larger marginal cost of maintaining epigenetic integrity relative to young individuals. These effects are likely to interact, such that the effect of old age is accentuated by low condition. Such experiments would make it possible to test for differential costs of investment in epigenetic paternal effects, and potentially make it possible to quantify such costs.

Concluding remarks

Establishing to what extent paternal effects transmitted through epigenetic factors function as costly and condition-dependent life-history traits requires additional theoretical and empirical work, and will necessitate overcoming some practical challenges. Progress will require an understanding of when investment in the epigenetic machinery occurs (i.e., are the key epigenetic systems built during juvenile development, or during the adult stage?), knowing what epigenetic factors are involved in influencing offspring quality in specific study species, as well as the ability to manipulate individual condition and investment in epigenetic factors that mediate paternal effects. Although the technology available for direct manipulation of epigenetic mechanisms is currently limited, it is progressing at a rapid rate (e.g., Frye et al. 2016; McDonald et al. 2016; Abudayyeh et al. 2016; Pulecio *et al.*, 2017). And while we have focused on the most widely studied epigenetic factors (DNA methylation, ncRNAs and chromatin structure), the sperm and semen also contain many other nongenetic factors (such as cytoplasmic and accessory-gland proteins) that are not conventionally regarded as forms of nutrient provisioning or categorized as instances of transgenerational epigenetic inheritance, but that could nonetheless influence offspring development.

Understanding the differential costs and condition dependence of nongenetic paternal effects mediated by epigenetic factors will make it possible to extend life history theory to encompass this poorly understood facet of male reproductive strategies. Understanding such effects will also shed light on a potentially important component of variation in offspring performance, and a potential factor in the evolution of female mate choice. Moreover, epigenetic paternal effects could provide a valuable opportunity to investigate the costs of

building, maintaining and deploying various types of epigenetic machinery—a question that remains almost entirely unexplored.

References

ABUDAYYEH, O.O., GOOTENBERG, J.S., KONERMANN, S., JOUNG, J., SLAYMAKER, I.M., COX, D.BT, *et al.* (2016). C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* **353**, 5571–5579.

ADLER, M.I., BONDURIANSKY, R. (2013). Paternal effects on offspring fitness reflect father's social environment. *Journal of Evolutionary Biololgy* **40**, 288–292.

AGRAWAL, A.F., WANG, A.D. (2008). Increased transmission of mutations by low-condition females: evidence for condition-dependent DNA repair. *PLoS Biology* **6**, 0389–0395.

AIKEN, C.E., OZANNE, J.L., TARRY-ADKINS, S.E. (2016). Transgenerational effects of maternal diet on metabolic and reproductive ageing. *Mammal Genome* **27**, 430–439.

AMIOTT, E.A., JAEHNING, J.A. (2006). Mitochondrial transcription is regulated via an ATP 'sensing' mechanism that couples RNA abundance to respiration. *Molecular Cell* **22**, 329–338.

ANDERSSON, M. (1982). Sexual selection, natural selection, and quality advertisement. *Biological Journal of the Linnean Society* **82**, 375–393.

ASHAPKIN, V. V., KUTAEVA, L.I., VANYUSHIN, B.F. (2017). Aging as an epigenetic phenomenon. *Current Genomics* **18**, 385–407.

AVILA, F.W., SIROT, L.K., LAFLAMME, B.A., RUBINSTEIN, C.D., WOLFNER, M.F. (2011). Insect seminal fluid proteins: identification and function. In: Berenbaum MR, Carde RT, Robinson GE (eds) *Annual Review of Entomology* **56**, 21–40.

BADYAEV, A.V., HILL, G.E. (2002). Paternal care as a conditional strategy: distinct reproductive tactics associated with elaboration of plumage ornamentation in the house finch. *Behavioral Ecology* **13**, 591–597.

BARBOSA, T.D., INGERSLEV, L.R., ALM, P.S., VERSTEYHE, S., MASSART, J., RASMUSSEN, M., *et al.* (2016). High-fat diet reprograms the epigenome of rat spermatozoa and transgenerationally affects metabolism of the offspring. *Molecular Metabolism* **5**, 184–197.

BEACH, S.R.H., DOGAN, M.V., LEI, M., CUTRONA, C.E., GERRARD, M., GIBBONS, F.X., *et al.* (2015). Methylomic aging as a window onto the

influence of lifestyle: tobacco and alcohol use alter the rate of biological aging. *Journal of the American Geriatrics Society* **63**, 2519–2525.

BELLIZZI, D., D'AQUILA, P., MONTESANTO, A., CORSONELLO, A., MARI, V., MAZZEI, B., *et al.* (2012). Global DNA methylation in old subjects is correlated with frailty. *Age* **34**, 169–179.

BHUTANI, N., BURNS, D.M., BLAU, H.M. (2011). DNA demethylation dynamics. *Cell* **146**, 866–872.

BINDER, N.K., HANNAN, N.J., GARDNER, D.K. (2012). Paternal diet-induced obesity retards early mouse embryo development, mitochondrial Aactivity and pregnancy health. *PLoS One* **7**. 1–10.

BINDER, N.K., MITCHELL, M., GARDNER, D.K. (2012). Parental diet-induced obesity leads to retarded early mouse embryo development and altered carbohydrate utilisation by the blastocyst. *Reproduction Fertilility and Development* **24**, 804–812.

BOGGS, C.L. (1981). Nutritional and life-history determinants of resource-allocation in holometabolous insects. *Am Nat* **117**: 692–709.

BOKS, M.P., MIERLO, H.C., VAN RUTTEN, B.P.F., RADSTAKE, T.R.D.J., WITTE, L.D.E., GEUZE, E., *et al.* (2015). Longitudinal changes of telomere length and epigenetic age related to traumatic stress and post-traumatic stress disorder. *Psychoneuroendocrinology* **51**, 506–512.

BONDURIANSKY, R., CREAN, A.J. (2017). What are parental condition-transfer effects and how can they be detected? *Methods in Ecology and Evolution* **9**, 1–7.

BONDURIANSKY, R., DAY, T. (2009). Nongenetic inheritance and its evolutionary implications. *Annuual Review of Ecology and Evolution Systematics* **40**, 103–125.

BONDURIANSKY, R., DAY, T. (2013). Nongenetic inheritance and the evolution of costly female preference. *Journal of Evolutionary Biology* **26**, 76–87.

BONDURIANSKY, R., HEAD, M. (2007). Maternal and paternal condition effects on offspring phenotype in *Telostylinus angusticollis* **20** (Diptera: Neriidae). *Journal of Evolutionary Biology* **20**, 2379–2388.

BONDURIANSKY. R., RUNAGALL-MCNAULL, A., CREAN, A.J. (2016). The nutritional geometry of parental effects: maternal and paternal macronutrient consumption and offspring phenotype in a neriid fly.

Functional Ecology **30**, 1675-1686.

BONILLA, M.M., ZEH, J.A., ZEH, D.W. (2016). An epigenetic resolution of the lek paradox. *Bioessays* **38**, 355–366.

BRAUN, K., CHAMPAGNE, F.A. (2014). Paternal influences on offspring development: behavioural and epigenetic pathways. *Journal of Neuroendocrinology* **26**, 697–706.

BREITLING, L.P., SAUM, K.U., PERNA, L., SCHOTTKER, B., HOLLECZEK, B., BRENNER, H. (2016). Frailty is associated with the epigenetic clock but not with telomere length in a German cohort. *Clinical Epigenetics* **8**, 1–8.

BRETMAN, A., FRICKE, C., WESTMANCOAT, J.D., CHAPMAN, T. (2016). Effect of competitive cues on reproductive morphology and behavioral plasticity in male fruitflies. *Behavioral Ecology* **27**, 452–461.

BURDGE, G.C., LILLYCROP, K.A. (2010). Nutrition, epigenetics, and developmental plasticity: Implications for understanding human disease. *Annual Review of Nutrition* **30**, 315–339.

BURDGE, G.C., SLATER-JEFFERIES, J., TORRENS, C., PHILLIPS, E.S., HANSON, M.A., LILLYCROP, K.A. (2007). Dietary protein restriction of pregnant rats in the F0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F1 and F2 generations. *British Journal of Nutrition* **97**, 435–439.

BURGESS, S.C., MARSHALL, D.J. (2014). Adaptive parental effects : the importance of estimating environmental predictability and offspring fitness appropriately. *Oikos* **123**, 769–776.

CARONE, B.R., FAUQUIER, L., HABIB, N., SHEA, J.M., HART, C.E., LI, R., *et al.* (2010). Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell* **143**, 1084–1096.

CHAMPAGNE, F.A. (2008). Epigenetic mechanisms and the transgenerational effects of maternal care. *Frontiers in Neuroendocrinology* **29**, 386–397.

COTTON, S., FOWLER, K., POMIANKOWSKI, A. (2004). Do sexual ornaments demonstrate heightened condition-dependent expression as predicted by the handicap hypothesis? *Proceeding of the Royal Society: Biological Science* **271**, 771–83.

CREAN, A.J., ADLER, M.I., BONDURIANSKY, R. (2016). Seminal fluid and mate

choice: new predictions. *Trends in Ecology and Evolution* **31**, 253–255.

CREAN, A.J., BONDURIANSKY, R. (2014). What is a paternal effect? *Trends in Ecology and Evolution* **29**, 554–559.

CREAN, A.J., DWYER, J.M., MARSHALL, D.J. (2013). Adaptive paternal effects? Experimental evidence that the paternal environment affects offspring performance. *Ecology* **94**, 2575–2582.

CREAN, A.J., KOPPS. A.M., BONDURIANSKY, R. (2014). Revisiting telegony: offspring inherit an acquired characteristic of their mother's previous mate. *Ecology Letters* **17**, 1545–1552.

CLUTTON-BROCK, T.H. (1991). *The Evolution of Parental Care*. Princeton University Press: Princeton, New Jersey.

DANCHIN, E., CHARMANTIER, A., CHAMPAGNE, F.A., MESOUDI, A., PUJOL, B., BLANCHET, S. (2011). Beyond DNA: integrating inclusive inheritance into an extended theory of evolution. *Nature Reviews Genetics* **12**, 475–486.

DELCURTO, H., WU, G., SATTERFIELD, M.C. (2013). Nutrition and reproduction : links to epigenetics and metabolic syndrome in offspring. *Currrnt Opinions in Clinical Nutrition Metabolic Care* **16**, 385–391.

DICK, K.J., NELSON, C.P., TSAPROUNI, L., SANDLING, J.K., AÏSSI, D., WAHL, S., *et al.* (2014). DNA methylation and body-mass index : a genome-wide analysis. *Lancet* **383**, 1990–1998.

DUALE, N., STEFFENSEN, I., ANDERSEN, J., BREVIK, A., BRUNBORG, G., LINDEMAN, B. (2014). Impaired sperm chromatin integrity in obese mice. *Andrology* **2**, 234–243.

DUCATEZ, S., BAGUETTE, M., STEVENS, V.M., LEGRAND, D., FR, H. (2012). Complex interactions betweem paternal and materanal effects: parental experience and age at reproduction affect fecundity and offspring performance in a butterfly. *Evolution* **66**, 3558–3569.

EATON, S.A., JAYASOORIAH, N., BUCKLAND, M.E., MARTIN, D.I.K., CROPLEY, J.E., SUTER, C.M. (2015). Roll over Weismann: extracellular vesicles in the transgenerational transmission of environmental effects. *Epigenomics* **7**, 1165–1171.

EBERHARD, W.G. (1997). Sexual selection by cryptic female choice in insects and arachnids. In: Chloe JC, Crepsi BJ (eds) *The Evolution of mating Systems in Insects and Arachnids,* Cambridge University Press: Cambridge, pp 32-57.

EBERHARD, W.G., CORDERO, C. (1995). Sexual selection by cryptic female choice on male seminal products - a new bridge between sexual selection and reproductive physiology. *Trends in Ecology and Evolution* **10**, 493–496.

EVANS, J.P., LYMBERY, R.A., WIID, K.S., RAHMAN, M., GASPARINI, C. (2017). Sperm as moderators of environmentally induced paternal effects in a livebearing fish. *Biology Letters* **13**, 10–13.

FITZPATRICK, J.L., LÜPOLD, S. (2014). Sexual selection and the evolution of sperm quality. *Molecular Human Reproduction* **20**, 1180–1189.

FRICKE, C., ADLER, M.I., BROOKS, R.C., BONDURIANSKY, R. (2015). The complexity of male reproductive success: effects of nutrition, morphology, and experience. *Behavioral Ecology* **26**, 617–624.

FRIESEN, C.R., POWERS, D.R., COPENHAVER, P.E., MASON, R.T. (2015). Size dependence in non-sperm ejaculate production is reflected in daily energy expenditure and resting metabolic rate. *Journal of Experimental Biology* **218**, 1410–1418.

FRYE, M., JAFFREY, S.R., PAN, T., RECHAVI, G., SUZUKI, T. (2016). RNA modifications: what have we learned and where are we headed? *Nature Reviews Genetics* **17**, 365–372.

FULLSTON, T., TEAGUE, E.M.C.O., PALMER, N.O., DEBLASIO, M.J., MITCHELL, M., CORBETT, M., *et al.* (2013). Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *Faseb Journal* **27**, 4226–4243.

GAAL, T., BARTLETT, M.S., ROSS, W., TURNBOUGH, C.L., GOURSE, R.L. (1997). Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. *Science* **278**, 2092–2097.

GAO, X., ZHANG, Y., BREITLING, L.P., BRENNER, H. (2016). Relationship of tobacco smoking and smoking-related DNA methylation with epigenetic age acceleration. *Oncotarget* **7**, 46878–46889.

GAPP, K., JAWAID, A., SARKIES, P., BOHACEK, J., PELCZAR, P., PRADOS, J., *et al.* (2014). Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nature Neuroscience* **17**, 667–9.

GODWIN, J.L., VASUDEVA, R., MICHALCZYK, L., MARTIN, O.Y., LUMLEY, A.J., CHAPMAN, T., *et al.* (2017). Experimental evolution reveals that sperm

competition intensity selects for longer, more costly sperm. *Evolution Letters* **1**, 102–113.

GRAFEN, A. (1990). Biological signals as handicaps. *Journal of Theoretical Biology* **144**, 517–546.

GRANDISON, R.C., PIPER, M.D.W., PARTRIDGE, L. (2009). Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. *Nature* **462**, 1061–1065.

GRANDJEAN, V., FOURRE, S., DE ABREU, D.A.F., DERIEPPE, M.A., REMY, J.J., RASSOULZADEGAN, M. (2015). RNA-mediated paternal heredity of diet-induced obesity and metabolic disorders. *Scientific Reports* **5**, 1–9.

GUERRERO-BOSAGNA, C., SETTLES, M., LUCKER, B., SKINNER, M.K. (2010). Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. *PLoS One* **5**, 1–17.

GUERRERO-BOSAGNA, C., SKINNER, M.K. (2014). Environmentally induced epigenetic transgenerational inheritance of male infertility. *Current Opinion in Genettics and Development* **26**, 79–88.

GWYNNE, D.T. (1988). Courtship feeding in katydids benefits the mating male's offspring. *Behaviour* **23**, 373–377.

GWYNNE, D.T., SIMMONS, L.W. (1990). Experimental reversal of courtship roles in an insect. *Nature* **346**, 172–174.

HEAD, M.L., JENNIONS, M.D., ZAJITSCHEK, S.R.K. (2016). Sexual selection: incorporating non-genetic inheritance. *Current Opinion in Behavioural Science* **12**, 129–137.

HORVATH, S. (2013). DNA methylation age of human tissues and cell types. *Genome Biology* **14**, 1–19.

HORVATH, S., ERHART, W., BROSCH, M., AMMERPOHL, O., SCHÖNFELS, W. V.O.N., AHRENS, M., *et al.* (2014). Obesity accelerates epigenetic aging of human liver. *Proceedings of the National Acadamy of Science* **111**: 15538–15543.

IWASA, Y., POMIANKOWSKI, A., NEE, S. (1991). The evolution of costly mate preferences II. The 'handicap' principle. *Evolution* **45**, 1431–1442.

JENKINS, T.G., CARRELL, D.T. (2012). The sperm epigenome and potential implications for the developing embryo. *Reproduction* **143**, 727–734.

JENSEN, N., ALLEN, R.M., MARSHALL, D.J. (2014). Adaptive maternal and paternal effects : gamete plasticity in response to parental stress. *Functional*

Ecology **28**, 724–733.

JIRTLE, R.L., SKINNER, M.K. (2007). Environmental epigenomics and disease susceptibility. *Nature Reviews Genetics* **8**, 253–62.

JUDGE, K.A., TING, J.J., GWYNNE, D.T. (2008). Condition dependence of male life span and calling effort in a field cricket. *Evolution* **62**, 868–878.

KAATI, G., BYGREN, L.O., PEMBREY, M., SJO, M. (2007). Transgenerational response to nutrition, early life circumstances and longevity. *European Journal of Human Genetics* **15**, 784–790.

KATO, Y., KANEDA, M., HATA, K., KUMAKI, K., HISANO, M., KOHARA,Y., *et al.* (2007). Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. *Human Molecular Genetics* **16**, 2272–2280.

KELLY, W.G. (2014). Transgenerational epigenetics in the germline cycle of *Caenorhabditis elegans*. *Epigenetics Chromatin* **7**, 1–17.

KELLY, N.B., ALONZO, S.H. (2009). Will male advertisement be a reliable indicator of paternal care, if offspring survival depends on male care? *Proceedings of the Royal Society: Biological Science* **276**, 3175–3183.

KITAMURA, A., MIYAUCHI, N., HAMADA, H., HIURA, H., CHIBA, H., OKAE, H., *et al.* (2015). Epigenetic alterations in sperm associated with male infertility. *Congenital Anomolies* **55**, 133–144.

KLOSIN, A., CASAS, E., HIDALGO-CARCEDO, C., VAVOURI, T., LEHNER, B. (2017). Transgenerational transmission of environmental information in *Caenorhabditis elegans*. *Science* **356**, 320–323.

KOKKO, H. (1999). Cuckoldry and the stability of biparental care. *Ecology Letters* **2**, 247–255.

KOKKO, H., JENNIONS, M.D. (2008). Parental investment, sexual selection and sex ratios. *Journal of Evolionary Biology* **21**, 919–948.

KOTIAHO, J.S. (2000). Testing the assumptions of conditional handicap theory: costs and condition dependence of a sexually selected trait. *Behavioral Ecology and Sociobiology* **48**, 188–194.

KOTIAHO, J.S. (2001). Costs of sexual traits: a mismatch between theoretical considerations and empirical evidence. *Biological Reviews* **76**, 365–376.

LAMBROT, R., XU, C., SAINT-PHAR, S., CHOUNTALOS, G., COHEN, T., PAQUET, M., *et al.* (2013). Low paternal dietary folate alters the mouse sperm

epigenome and is associated with negative pregnancy outcomes. *Nature Communications* **4**, 1–13.

LINKLATER, J.R., WERTHEIM, B., WIGBY, S., CHAPMAN, T. (2007). Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in Drosophila melanogaster. *Evolution* **61**, 2027–2034.

LÜPOLD, S., MANIER, M.K., PUNIAMOORTHY, N., SCHOFF, C., STARMER, W.T., LUEPOLD, S.H.B., *et al.* (2016). How sexual selection can drive the evolution of costly sperm ornamentation. *Nature* **533**, 535–538.

MACARTNEY, E.L., CREAN, A.J., BONDURIANSKY, R. (2017). Adult dietary protein has age- and context-dependent effects on male post-copulatory performance. *Journal of Evolutionary Biology* **38**, 42–49.

MANIKKAM, M., GUERRERO-BOSAGNA, C., TRACEY, R., HAQUE, M., SKINNER, M.K. (2012). Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. *PLoS One* **7**.

MAPPES, J., ALATALO, R.V., KOTIAHO, J., PARRI, S. (1996). Viability costs of condition-dependent sexual male display in a drumming wolf spider. *Proceedings of the Royal Society: Biological Science* **263**, 785–789.

MARCOTTE, M., DELISLE, J., MCNEIL, J.N. (2007). Effects of different male remating intervals on the reproductive success of Choristoneura rosaceana males and females. *Journal of Insect Physiology* **53**, 139–145.

MARRÉ, J., TRAVER, E.C., JOSE, A.M. (2016). Extracellular RNA is transported from one generation to the next in *Caenorhabditis elegans*. *PNAS* **113**, 12496–12501.

MARSHALL, D.J., ULLER, T. (2007). When is a maternal effect adaptive? *Oikos* **116**, 1957–1963.

MARTTILA, S., KANANEN, L., HÄYRYNEN, S., JYLHÄVÄ, J., NEVALAINEN, T., HERVONEN, A., *et al.* (2015). Ageing-associated changes in the human DNA methylome: genomic locations and effects on gene expression. *BMC Genomics* **16**, 1–17.

MAYNARD SMITH, J. (1977). Parental investment: a prospective analysis. *Animal Behaviour* **25**, 1–9.

MCDONALD, J.I., CELIK, H., ROIS, L.E., FISHBERGER, G., FOWLER, T., REES, R., *et al.* (2016). Reprogrammable CRISPR/Cas9-based system for

inducing site-specific DNA methylation. *Biology Open* **5**, 866–74.

MICHAUD, J.P., BISTA, M., MISHRA, G., SINGH, O. (2013). Sexual activity diminishes male virility in two Coccinella species: consequences for female fertility and progeny development. *Bulletin of Entomological Research* **103**, 570–577.

MILEKIC, M.H., XIN, Y., DONNELL, A.O., KUMAR, K.K., MALASPINA, D., MOORE, H., *et al.* (2015). Age-related sperm DNA methylation changes are transmitted to offspring and associated with abnormal behavior and dysregulated gene expression. *Molecular Psychiatry* **20**, 995–1001.

MILLER, D., BRINKWORTH, M., ILES, D. (2010). Paternal DNA packaging in spermatozoa: More than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction* **139**, 287–301.

MIRHOSSEINI, M.A., MICHAUD, J.P., JALALI, M.A., ZIAADDINI, M. (2014). Paternal effects correlate with female reproductive stimulation in the polyandrous ladybird *Cheilomenes sexmaculata*. *Bulletin of Entomological Research* **104**, 480–485.

MISHRA, G., OMKAR. (2006). Ageing trajectory and longevity trade-off in an aphidophagous ladybird, Propylea dissecta (Coleoptera: Coccinellidae). *European Journal of Entomology* **103**, 33–40.

MITCHELL, M., STRICK, R., STRISSEL, P.L., DITTRICH, R., MCPHERSON, N.O., LANE, M., *et al.* (2017). Gene expression and epigenetic aberrations in F1-placentas fathered by obese males. *Molecular Reproduction and Development* **84**, 316–328.

MOLLER, A.P., DELOPE, F. (1994). Differential costs of a secondary sexual character - an experimental test of the handicap principle. *Evolution* **48**, 1676–1683.

NG, S.F., LIN, R.C.Y., LAYBUTT, D.R., BARRES, R., OWENS, J.A., MORRIS, M.J. (2010). Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. *Nature* **467**, 963–966.

NUR, N., HASSON, O. (1984). Phenotypic plasticity and the handicap principle. *J Theoretical Biology* **110**, 275–297.

OKANO, M., BELL, D.W., HABER, D.A., LI, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99**, 247–257.

PERRY, J.C., ROWE, L. (2010). Condition-dependent ejaculate size and composition in a ladybird beetle. *Proceedings of the Royal Society: Biological Science* **277**, 3639–3647.

PERRY, J.C., TSE, C.T. (2013). Extreme costs of mating for male two-spot ladybird beetles. *PLoS One* **8**. 1–5.

PULECIO, J., VERMA, N., MEJIA-RAMIRES, E., HUANGFU. D., RAYA, A. (2017). Protocol review CRISPR / Cas9-based engineering of the epigenome. *Cell Stem Cell* **21**, 431–447.

RANDO, O.J. (2012). Daddy Issues: paternal effects on phenotype. *Cell* **151**, 702–708.

RANDO, O.J. (2016). Intergenerational transfer of epigenetic information in sperm. *Cold Spring Harbour Perspective Biology* **6**, 1–14.

RASSOULZADEGAN, M., GRANDJEAN, V., GOUNON, P., VINCENT, S., GILLOT, I., CUZIN, F. (2006). RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. *Nature* **441**, 469–474.

REQUENA, G.S., ALONZO, S.H. (2017). Sperm competition games when males invest in paternal care. *Proceedings of the Royal Society: Biological Science* **284**.

RODGERS, A.B., MORGAN, C.P., LEU, N.A., BALE, T.L. (2015). Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. *Proceedings of the National Academy of Science* **112**, 13699–13704.

ROFF, D.A., FAIRBAIRN, D.J. (2007). The evolution of trade-offs: where are we? *Journal of Evolutionary Biology* **20**, 433–447.

ROWE, L., HOULE, D. (1996). The lek paradox and the capture of genetic variance by condition dependent traits. *Proceedings of the Royal Society: Biological Science* **263**, 1415–1421.

ROYLE, N.J., SMISETH, P.T., KÖLLIKER, M. (2012). *The Evolution of Parental Care*. Oxford University Press.

RUTOWSKI, R.L. (1979). Butterfly as an honest salesman. *Animal Behaviour* **27**, 1269–1270.

SAZE, H., MITTELSTEN SCHEID, O., PASZKOWSKI, J. (2003). Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. *Nature Genetics* **34**, 65–69.

SEONG, K.H., LI, D., SHIMIZU, H., NAKAMURA, R., ISHII, S. (2011). Inheritance

of stress-induced, ATF-2-dependent epigenetic change. *Cell* **145**, 1049–1061.

SHELDON, B.C. (2002). Relating paternity to paternal care. *Philosophical Transactions of the Royal Society: Biological Science* **357**, 341–350.

SKINNER, M.K. (2016). Epigenetic transgenerational inheritance. *Nature Reviews* **12**, 68–70.

SKINNER, M.K., GUERRERO-BOSAGNA, C., HAQUE, M.M. (2015). Environmentally induced epigenetic transgenerational inheritance of sperm epimutations promote genetic mutations. *Epigenetics* **10**, 762–771.

SOUBRY, A. (2015). Epigenetic inheritance and evolution: a paternal perspective on dietary influences. *Progress in Biophysics and Molecular Biology* **118**, 79–85.

STEARNS, S.C. (1989). Trade-offs in life history evolution. *Functional Ecology* **3**, 259–268.

STIVER, K.A., ALONZO, S.H. (2009). Parental and mating effort: Is there necessarily a trade-off? *Ethology* **115**, 1101-1126.

STOECKIUS, M., GRÜN, D., RAJEWSKY, N. (2014). Paternal RNA contributions in the *Caenorhabditis elegans* zygote. *EMBO J* **33**, 1740–1750.

THORNHILL, R. (1976). Sexual selection and paternal investment in insects. *American Naturalist* **110**, 153–163.

TRIVERS, R.L. (1972). Parental investment and sexual selection. In: B Campbell (ed)*, Sexual Selection and the Descent of Man*. Aldeline Press: Chicago, pp 136–179.

VASSOLER, F.M., WHITE, S.L., SCHMIDT, H.D., SADRI-VAKILI, G., PIERCE, R.C. (2012). Epigenetic inheritance of a cocaine-resistance phenotype. *Nature Neuroscience* **16**, 42–47.

VASUDEVAN, S., TONG, Y., STEITZ, J.A. (2007). Switching from repression to activation: microRNAs can up-regulate translation. *Science* **318**, 1931–1934.

WANG, Y., LIU, H., SUN, Z. (2017). Lamarck rises from his grave: parental environment-induced epigenetic inheritance in model organisms and humans. *Biological Reviews* **92**, 2084–2111.

WELLEN, K.E., HATZIVASSILIOU, G., SACHDEVA, U.M., BUI, T.V., CROSS, R., THOMPSON, C.B., *et al.* (2009). ATP-citrate lyase links cellular metabolism

to histone acetylation. *Science* **324**, 1076–1080.

WEST, H.E.R., CAPELLINI, I. (2016). Male care and life history traits in mammals. *Nature Communications* **7**, 1–10.

WEYRICH, A., LENZ, D., JESCHEK, M., CHUNG, T.H., RÜBENSAM, K., GÖRITZ, F., *et al.* (2016). Paternal intergenerational epigenetic response to heat exposure in male Wild Guinea pigs. *Molecular Ecology* **25**, 1729–1740.

WICKLER, W. (1985). Stepfathers in insects and their pseudo-parental investment. *Zeitschrift für Tierpsychologie* **69**, 72–78.

WIGBY, S., PERRY, J.C., KIM, Y.H., SIROT, L.K. (2016). Developmental environment mediates male seminal protein investment in *Drosophila melanogaster*. *Functional Ecology* **30**, 410–419.

ZAJITSCHEK, F., ZAJITSCHEK, S., MANIER, M. (2017). High-protein paternal diet confers an advantage to sons in sperm competition. *Biology Letters* **13**. 1–5.

ZERA, A.J., HARSHMAN, L.G. (2001). The Physiology of Life History Trade-Offs in Animals. *Annual Review in Ecology and Systematics* **32**, 95–126.

Chapter Five

MALE PRUDENCE MITIGATES COSTS OF MATING ON POST-COPULATORY PERFORMANCE IN THE NERIID FLY *TELOSTYLINUS ANGUSTICOLLIS*

Erin L Macartney, Russell Bonduriansky, Angela J Crean

ELM conceived, designed and ran the experiment, analysed the data and wrote the manuscript. RB and AJC provided valuable advice throughout all phases of this manuscript.

Abstract

Frequent mating can deplete ejaculate components, limiting male fecundity. The ejaculate can also contain molecular signals that mediate non-genetic paternal effects, raising the possibility that frequent mating may also influence the presence and/or magnitude of paternal effects. Ejaculate components are often condition-dependent whereby males in low condition are less able to invest in such traits, and this suggests that ejaculate depletion may also be condition-dependent. In this study, we examined if frequent mating induced a condition-dependent reduction in male fecundity and ability to induce non-genetic paternal effects on offspring traits in the neriid fly, *Telostylinus angusticollis*. We manipulated male condition by altering larval nutrient availability and allowed some males to mate frequently while others remained virgins. We then measured aspects of male fecundity (male-induced egg output and egg hatching success), and non-genetic paternal effects on offspring traits likely related to fitness in the wild (egg-to-adult viability and offspring body size) when paired with a novel female. We predicted that the previously mated males would have reduced fecundity and offspring quality when paired with a novel female and this reduction would be greater in males fed a nutrient-poor larval diet. Interestingly, we did not detect an effect of mating, nor an effect of larval diet on male fecundity or offspring traits. However, we found that males appeared to strategically alter their reproductive behaviour when encountering novel females by reducing their mating rate. Therefore, male neriid flies appear to be able to mate many times without suffering a reduction in post-copulatory performance, but this effect is likely mediated by a reduction in mating rate and reflective of male prudence with ejaculate expenditure.

Introduction

Frequent mating can decrease male fecundity (i.e., number of progeny sired) by reducing sperm and non-sperm components of the ejaculate (e.g., Dewsbury, 1982; Preston *et al.*, 2001; Torres-Vila and Jennions, 2005; Marcotte *et al.*, 2007; Wigby *et al.*, 2009; Reinhardt *et al.*, 2011; Muller *et al.*, 2016; Hopkins *et al.*, 2019). For example, male Soay rams (*Ovis aries*) become sperm depleted over the course of the breeding season and suffer a reduction in the number of lambs sired (Preston *et al.*, 2001). This reduction in fecundity can also occur rapidly with frequent mating in some species. For example, three to five matings is enough to decrease accessory gland size, testes size, male-induced egg-output and the number of progeny sired in *Drosophila melanogaster* (Hihara, 1981; Linklater *et al.*, 2007; Hopkins *et al.*, 2019). However, male fitness is not only determined by the quantity of offspring produced, but can also be affected by the quality of offspring produced (Maynard Smith, 1977; Clutton-Brock, 1991; Sheldon, 2002; Crean and Bonduriansky, 2016).

Males of many species can produce offspring of different quality through non-genetic mechanisms (e.g. Rando, 2016; Wang *et al.*, 2017). Males of some species transfer large and nutrient-rich ejaculates such as costly spermatophores or nuptial gifts (reviewed in Vahed, 1998), and a few studies on such species have demonstrated that frequent mating can reduce aspects of offspring quality. For example, Michaud *et al.* (2013) found that larval survival decreased and development time increased after frequent mating in two ladybird species (*Coccinella septempunctata* and *Coccinella transversalis*), and Mirhosseini *et al.* (2014) demonstrated that frequently mated males had decreased offspring weight in the ladybird *Cheilomenes sexmaculata*. However, even in species that lack nutrient-rich spermatophores, males can still influence the quality of their offspring via nongenetic paternal effects (e.g. Bonduriansky and Head, 2007; Delcurto *et al.*, 2013; Crean, Adler, & Bonduriansky, 2016; Evans *et al.*, 2017; Zajitschek *et al.*, 2017). Non-genetic paternal effects in these species may be conferred through epigenetic factors (i.e. differences in DNA methylation, chromatin structure or non-coding RNAs) (reviewed in Rando, 2012), or through non-nutritional proteins and peptides in the seminal fluid (Garcia-Gonzalez & Simmons, 2010; Avila *et al.*, 2011). Yet, the costs of

mating on offspring quality in such species are yet to be explored (see Macartney, Crean, *et al.*, 2018).

If ejaculate-borne factors that enhance male fecundity and offspring quality are depleted with mating, we may expect the rate of depletion to be condition-dependent. Many sperm and seminal traits have been shown to be condition-dependent, whereby males in high condition (i.e. having more energetic resources available to them) are able to invest more in such traits (reviewed in Macartney *et al.*, 2019). Male investment in offspring quality may also be condition-dependent (Delcurto *et al.*, 2013; Bonduriansky & Crean, 2017; Evans *et al.*, 2017; Zajitschek *et al.*, 2017; Macartney *et al.*, 2018a). If high-condition males are better able to invest in sperm and seminal traits as well as produce better quality offspring, they may also be better able to avoid depletion of sperm and seminal fluid components with frequent mating. For example, a recent study by Polak *et al.* (2017) found that calorie-restricted *Drosophila melanogaster* sired offspring with greater embryo mortality if they had mated once previously. This suggests that, for *D. melanogaster* males in low condition, even one mating is enough to reduce offspring quality.

Here, we investigate the costs of mating on male ability to induce female oviposition and egg hatching success (fecundity), and intergenerational paternal effects on egg-to-adult viability and offspring body size (traits that are likely to be positively associated with fitness in natural populations), using the neriid fly *Telostylinus angusticollis*. Males of this species do not transfer large, nutrient rich ejaculates, but previous studies have demonstrated non-genetic paternal effects on egg-to-adult viability and offspring body size (Bonduriansky & Head, 2007; Adler & Bonduriansky, 2013; Macartney, Crean, & Bonduriansky, 2017), and these effects appear to be conferred through components in the seminal fluid rather than epigenetic factors in the sperm (Crean, Kopps, & Bonduriansky, 2014). While we do not have precise estimates of mating rates in the wild for *T. angusticollis*, both females and males mate multiply in captivity. Therefore, males may experience depletion of sperm and seminal fluid components with frequent mating.

Materials and Methods

We carried out two experiments. In Experiment 1, we manipulated male condition by rearing males on either a 'nutrient-rich' or 'nutrient-poor' larval diet, and then randomly allocated them to either a 'mated' or 'control' group (hereafter referred to as the 'mating history assay'). Mated males were able to mate with five standardised females over a period of three hours, whereas control males were exposed to female cues for the same time period but prevented from mating. Males were then paired with a novel, virgin, assay female to measure latency of female oviposition, egg hatching success, egg-to-adult viability and offspring body size. We predicted that male fecundity and ability to confer non-genetic paternal effects on offspring traits would be reduced in previously mated males, and especially in males reared on the nutrient-poor diet. However, some costs of mating might have been undetectable in Experiment 1 because males from both treatments might have had enough ejaculate resources left over to achieve maximum fecundity and invest in offspring in a single mating with a novel female. Males might also have shown renewed mating and post-copulatory investment in a single novel female (e.g., Dewsbury, 1981; Pizzari *et al.*, 2003), thus preventing detection of a cost of mating. Therefore, in Experiment 2, male mating history was manipulated as before but males were subsequently given access to five novel, virgin, assay females, and we asked whether males that had previously mated many times would show a reduction in mating frequency and fecundity when given the opportunity to mate with multiple novel females.

Experiment 1

Rearing of experimental flies

40 male and 40 female flies were collected from Fred Hollows Reserve, Coogee, Sydney (33.91° S, 151.25° E) in March 2016. Flies were cultured for two generations prior to the experiment in a standardised environment, then 40 randomly selected males and females (F0) were paired to collect eggs for a split-brood experimental design. Pairs were housed in 250ml containers with damp cocopeat for hydration, fed sugar and yeast and provided pre-moulded 'rich' larval food for oviposition.

From each F0 pair, 20 randomly selected eggs were transferred to 100g of 'nutrient rich' larval diet, and 20 eggs were transferred to 100g of 'nutrient poor' larval diet to obtain focal males (F1) of 'high' and 'low' condition from each family. The rich larval diet consisted of 32g soy protein powder (Nature's Way brand, Pharm-a-care Pty. Ltd., Warriewood, NSW, Australia) and 89g brown sugar (brown sugar; Coles brand, Bundaberg, Australia) per 1 litre of coco-peat, and was hydrated with 600ml of water. The poor larval diet consisted of 5.5g protein and 14.8g brown sugar per litre of coco-peat and hydrated with 600ml of water. These diets were chosen to create flies of significantly different condition (see Sentinella, Crean, & Bonduriansky, 2013). We obtained 21 full-sib F1 families comprising of individuals reared on both the rich and the poor larval diet. A further 2000 eggs were transferred from the stock population to the rich larval diet (150g/50 eggs) to create females for the mating assay treatments and for the reproductive performance assays.

Immediately after emergence of adult F1 flies, two randomly selected males from the same larval diet and family were paired together in a 250ml container until the mating history assay. This was done because isolated males fail to undergo normal reproductive development (ELM and AJC, unpublished data). Adult males were provided with sugar but not protein to reduce the opportunity for replenishment of depleted ejaculate reserves as adult dietary protein has been shown to be important for ejaculate synthesis is other Dipteran species (e.g., Droney, 1998). Adult females were collected immediately after emergence, housed in groups of 10 in 1L containers, and fed sugar and yeast. All larval and adult flies were watered periodically and were kept at a constant temperature of 25° Celsius with a 12-hour light/dark cycle.

Male mating history assay

Focal males were between 15 and 20 days old (18.49 ± 1.16 days) at the time of the mating history assay. One randomly selected male from each pair was allocated to the 'mated' treatment group and the other male was allocated to the 'control' group (n=42 per mated and control group). Males in the mated treatment were each placed in a 1L container with five virgin females for 3

hours. As males will mate multiply with the same female (Macartney, 2015), and other studies on Diptera have found significant costs of mating after mating 3-5 times (e.g., Hihara, 1981; Linklater *et al.*, 2007; also see Hopkins *et al.*, 2019), five females were considered enough to detect costs of mating. The number of matings in the first hour was recorded for each male in the mating treatment and then every following half hour with half hour breaks in between observations. This was to test for differences in observed mating number between males of differing condition. Two males were not observed mating during the observation period, and so these males and their control group siblings were excluded from the reproductive performance assay. Control males were transferred individually to scintillation vials with a mesh lid and placed in the centre a of 1L container housing five virgin females for 3h. This allowed the males to receive visual and pheromonal cues through the mesh but prevented mating.

Male reproductive performance

After the 3h mating history assay period, focal males from the mated and control treatments were paired with a new virgin female (assay female) in a scintillation vial until mating was achieved. All assay females were standardised for age and diet. For logistical reasons, exact time to mating was not recorded, and males were categorised as 'early mating' (<30 mins until mating) or 'delayed mating' (>30 mins until mating) for analysis of 'latency to mate'. Eight out of 80 males took longer than 90 mins to initiate mating (see Results), and this was recorded and included in the latency to mate analysis as 'delayed mating'. However, these males were excluded from the reproductive performance assays of fecundity and offspring traits because their latency to mating was assumed sufficient to restore ejaculate stores.

After mating, the pairs were immediately separated, and the assay females were transferred individually to 250ml containers with oviposition medium (pre-moulded rich larval food) to measure male fecundity and non-genetic paternal effects on offspring (F2) traits.

The latency of female oviposition was measured (in days), and 20 randomly selected eggs were collected and placed on damp filter paper (to facilitate quantification of egg hatching success) on top of 100g of poor larval diet (as non-genetic effects of paternal condition may be more pronounced when offspring are reared on a nutrient-poor larval diet (Bonduriansky & Head, 2007)).

After 42 hours, the number of eggs to have hatched was recorded. Hatched eggs were identified as empty egg-shells under a Leica M60 stereo-microscope (Leica Microsystems, Heerbrugg, Switzerland). Larvae were then left to develop at a constant temperature of 25°C with a light-dark cycle of 12 hours and watered ad libitum. Egg-to-adult viability was determined by the number of F2 adult flies to emerge out of the 20 eggs transferred to measure egg hatching success.

Morphometric data

To quantify body size, thorax lengths of focal F1 males and the assay females that they were paired with, and of five randomly selected offspring of each sex per F2 brood (where possible), were measured using ImageJ (Rasband, 2015) from photos taken under 6.3 x magnification. Photos were taken using a Leica DFC420 camera mounted on a Leica MS5 microscope.

Experiment 2

Rearing of experimental flies

New fly stocks were collected from Fred Hollows Reserve (30 males and 30 females) in February 2018 and maintained for one generation.

To obtain experimental flies, 4300 eggs were collected from the laboratory stock population and all larvae were reared on the rich larval diet (see above). Larval diet was not manipulated in Experiment 2 because we did not detect any effects of larval diet in Experiment 1, suggesting that males of both high and low condition invest equally in post-copulatory performance. Therefore, Experiment 2 aimed to further explore potential costs of mating history on

post-copulatory performance without the need to consider dietary effects on male condition. 50 eggs were transferred to 150g of larval food in each of 86 larval containers. All flies were reared as described above in Experiment 1.

Male mating history assay

Focal males aged between 15 and 18 days old (16.95± 0.124) were randomly allocated to a mated treatment or control group (n = 40 per treatment), as in Experiment 1. Number of matings was recorded for males from the mated treatment during the first 45 minutes and the last 45 minutes of the 3-h treatment period.

Male reproductive performance

After the treatment period, focal males from the mated and control groups were placed in another 1L container with five new standardised virgin females (assay females) for 45 mins and the total number of matings across all five females was recorded. It must be noted that we originally intended to record which females the males mated with by marking the females. However, the markings disappeared during the reproductive performance assay, allowing us to record total matings but not how these matings were distributed across the five females.

After the assay period, the five assay females per male were placed in a 1L container with a large oviposition dish in order to measure each male's fecundity across the five females. The number of eggs laid by the females was counted after 72 hours (egg output), and then 20 randomly selected eggs were collected and placed on top of damp filter paper on top of a Petri dish filled with damp coco-peat and hatched vs unhatched eggs were recorded as per Experiment 1. The number of eggs laid as opposed to the latency for females to lay eggs as per Experiment 1 was measured due to logistical reasons. Most females laid eggs very rapidly in Experiment 2 compared to in Experiment 1, substantially reducing the variation in latency to lay eggs between individuals and between mating history treatments. However, both measures of fecundity are likely to be influenced by components in the male ejaculate (see Chapman 2001). We were unable to quantify transgenerational effects on offspring due

to very low F2 emergence (~3% of larvae eclosed into adults) across both treatments in this experiment.

Morphometric data

Measurements of focal male thorax length were taken as per Experiment 1.

Statistical analyses for Experiment 1 and 2

All analyses were completed in R version 3.3.2, using the *lme4* (Bates *et al.*, 2015) and *LmerTest* (Kuznetsova, Brockhoff, & Rune, 2017) packages. *LmerTest* calculates *p* values for Gaussian mixed effects models based on the Satterthwaite approximation for denominator degrees of freedom (Schaalje, McBride, & Fellingham, 2002).

For Experiment 1, effects of larval diet on male thorax length were analysed using a linear mixed-effects model with larval diet as the fixed effect and family as the random effect. The observed number of times mated treatment males mated in the 3h treatment period was analysed using a generalised linear mixed-effects model with a Poisson distribution, larval diet as a fixed effect, and family and observation level (to correct for over-dispersion) as random effects. Male mating latency with the new assay female (early mating = 1 versus delayed mating = 0), egg hatching success (hatched eggs = 1 versus not-hatched = 0), and egg-to-adult viability (emerged offspring = 1 versus not-emerged offspring = 0) were analysed with generalised linear mixed-effects models with binomial distributions. The latency for the assay females to lay eggs was analysed with a generalised linear mixed-effects model with a Poisson distribution, and offspring body size was analysed using linear mixed-effects models with Gaussian distributions. In the latter analysis, male and female offspring were pooled and offspring body size (thorax length) was standardised within offspring sex because there were no *a priori* predictions of treatment on male or female offspring body size, and there were no significant interactions of treatments and offspring sex on offspring body size. Models on male mating latency, female latency to lay eggs, egg hatching success, egg-to-adult viability, and offspring size included main effects and an interaction term of paternal larval diet and mating history, as well as male thorax length (standardised within larval diets to avoid co-linearity between the categorical predictor "larval

diet" and the continuous predictor "body size") and assay female thorax length as co-variates. Family was included as a random effect for all analyses and an observation level random effect was included in the male mating latency, egg hatching and egg-to-adult viability analyses to correct for overdispersion.

For Experiment 2, differences between mated and control males in the total number of observed matings across the five assay females after the mating history assay were analysed as generalised linear models with a Poisson distribution. A decrease in mating rate (i.e. a reduction in mating number over time) was analysed using a generalised linear model with a Poisson distribution, with observation period (i.e. the two observation periods during the mating treatment and the observation period during the reproductive performance assay) as the main effect. Effects of male mating history on fecundity (egg output and egg hatching success) were analysed using generalised mixed effects models with treatment as a fixed effect, male thorax length (unstandardized as all males were reared on the same larval diet) as a covariate and individual ID as a random effect to correct for over-dispersion. Egg output was analysed with a Poisson distribution and egg hatching success was analysed with a binomial distribution (hatched eggs = 1 versus not-hatched = 0). Intext descriptive statistics are written as means and standard errors.

Results

Experiment 1

Mating history assay

Males reared on the rich larval diet were significantly larger than males reared on the poor larval diet (rich larval diet: 1.706mm ± 0.065; poor larval diet: 1.263mm ± 0.063; estimate = 0.418, $p < 0.001$). During the mating treatment, males reared on the rich larval diet mated significantly more (7.72 ± 0.794 matings) than the males reared on the poor larval diet (4.48 ± 0.491 matings; estimate = 0.572, $p < 0.001$).

During the mating treatment, 16 out of 40 males were not observed mating in the last 30 minutes of the treatment period (7/16 of which were from the poor

larval diet and 9/16 from the rich larval diet). This suggests that the mating manipulation was successful at depleting male ejaculate reserves in at least some of the males across both larval diet treatments.

Reproductive performance assay

Males from the mating treatment took longer to mate with the novel assay female ('mating latency') than control males. Likewise, males from the poor larval diet had a longer mating latency than did males from the rich larval diet. However, there was no significant interaction of mating treatment and larval diet on mating latency (Table 1). 8 out of 80 males took > 90 minutes to initiate mating and, of these, 7/8 were from the mated treatment and 6/8 were from the poor larval diet.

There were no significant main effects or interactions of mating history and larval diet on any of the measures of fecundity or offspring traits (latency to lay eggs, egg hatching success, egg-to-adult viability or offspring body size) (Table 1, Fig. 1). Larger males (standardised within larval diet treatment groups) had increased egg hatching success and larger females produced significantly larger offspring.

Experiment 2

Mated treatment males suffered a significant reduction in mating rate over the three observation periods (estimate -0.441, $p < 0.001$) (Fig. 2).

Control males mated significantly more with the assay females (2.850 ± 0.295 matings across the five assay females) compared to males from the mated treatment (1.231 ± 0.233 matings; estimate =1.619, $p < 0.001$) (Fig. 2).

There was no effect of male mating history on egg output or egg hatching success (Table 2). Male thorax length was positively correlated with egg output and egg hatching success.

Table 1. Effects of larval diet, mating history and co-variates on mating latency, latency to lay eggs, egg hatching success, egg-to-adult viability and offspring body size in Experiment 1. Values in bold indicate a *p* value < 0.05.

	Mating latency		Latency to lay eggs		Egg hatching		Egg-to-adult viability		Offspring body size	
	Estimate	*p* value	Estimate	*p* value	Estimate	*p* value	Estimate	*p* value	Estimate	*p* value
Intercept	-4.904	0.673	0.733	0.689	14.510	0.215	-6.654	0.675	**-9.462**	**0.045**
Larval diet	**1.878**	**0.036**	0.134	0.332	0.152	0.875	1.825	0.160	0.126	0.462
Mating history	**2.113**	**0.022**	-0.033	0.828	0.606	0.528	-0.076	0.956	-1.751	0.083
Larval diet x mating history	-1.194	0.370	0.135	0.493	-1.544	0.254	-2.102	0.261	0.210	0.834
Male thorax length (standardised within larval diet)	-0.363	0.293	-0.087	0.235	**1.190**	**0.009**	0.245	0.601	-1.085	0.291
Assay female thorax length	2.194	0.742	0.668	0.495	-8.612	0.169	0.688	0.937	**5.030**	**0.044**

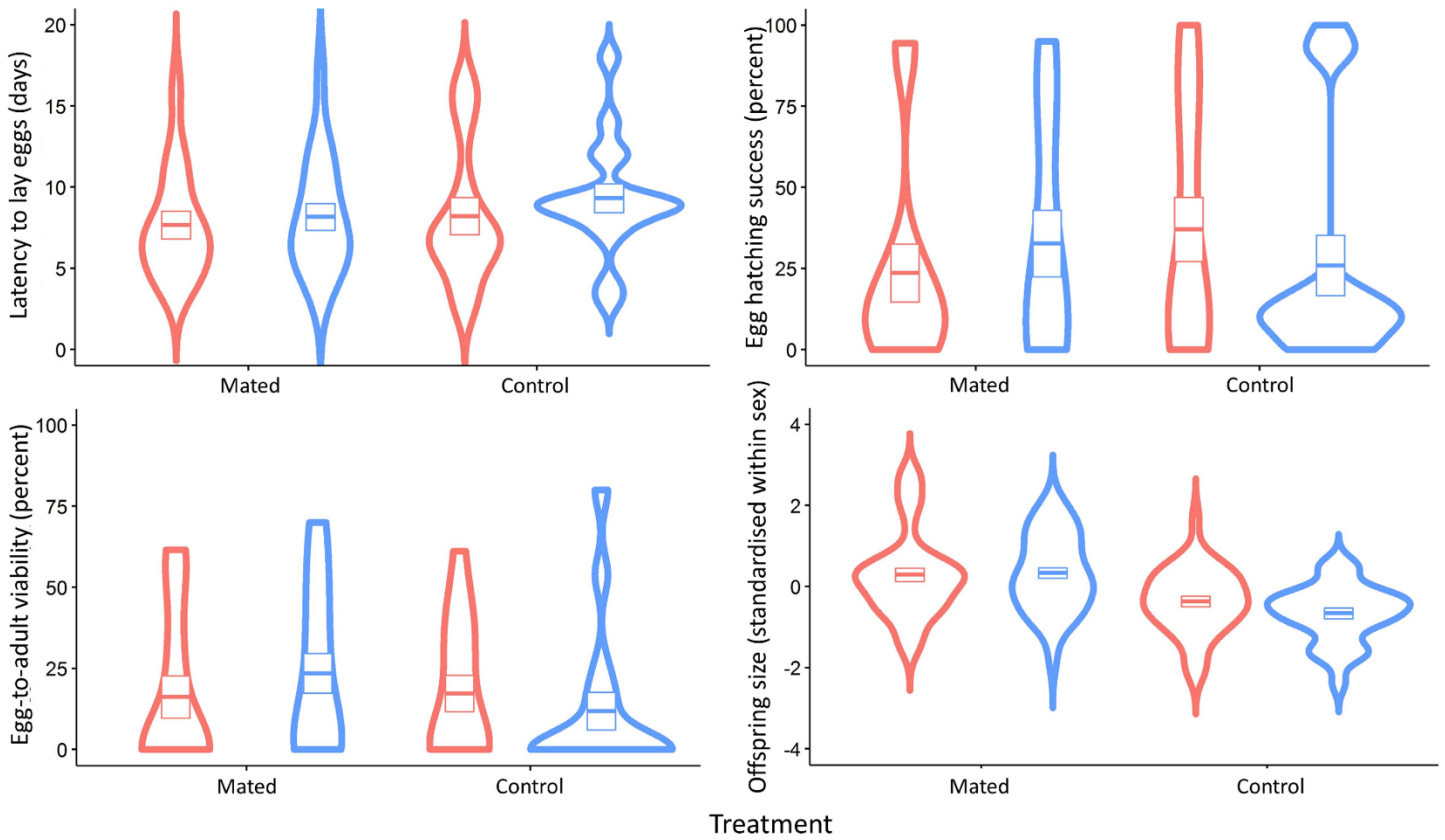


Figure 1. Violin plots showing the effects of the mated and control treatments, and the poor larval diet (red) and rich larval diet (blue) on male fecundity and offspring traits in Experiment 1. Offspring size is standardised within sex. Bars represent mean ± SE.

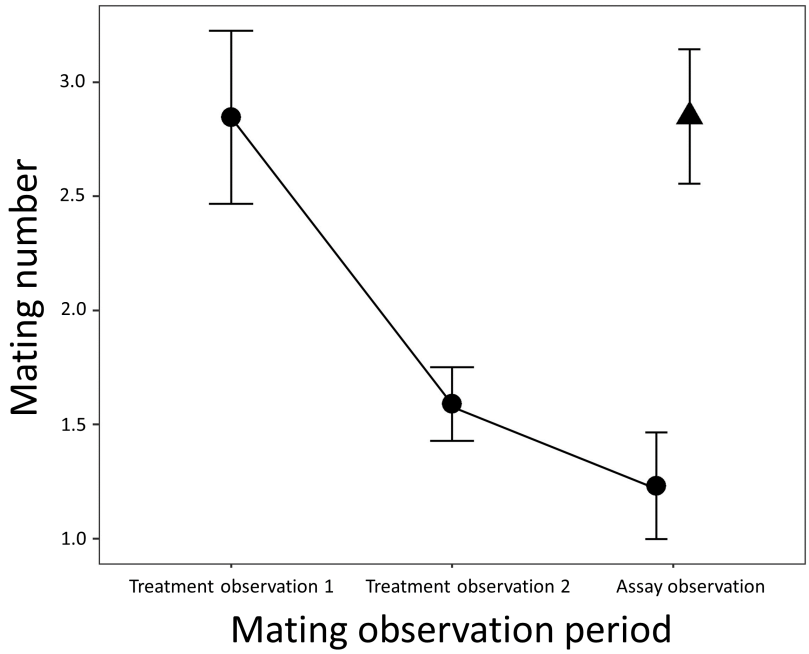


Figure 2. The observed mean ± SE of the total number of matings across five females in each observation period in Experiment 2. Males in the mated treatment were observed twice while in the treatment, and both control and mated males were observed with five novel females during the assay period. Mated treatment = circles, control = triangle.

Table 2. Effects of male mating history on egg output and egg hatching success with male thorax length as a co-variate from Experiment 2. Values in bold indicate a *p* value < 0.05.

	Egg output		Egg hatching	
	Estimate	*p* value	Estimate	*p* value
Intercept	**1.179**	**0.017**	**-7.373**	**0.006**
Mating history	0.058	0.098	-0.083	0.664
Male thorax length	**0.483**	**<0.001**	**1.867**	**0.011**

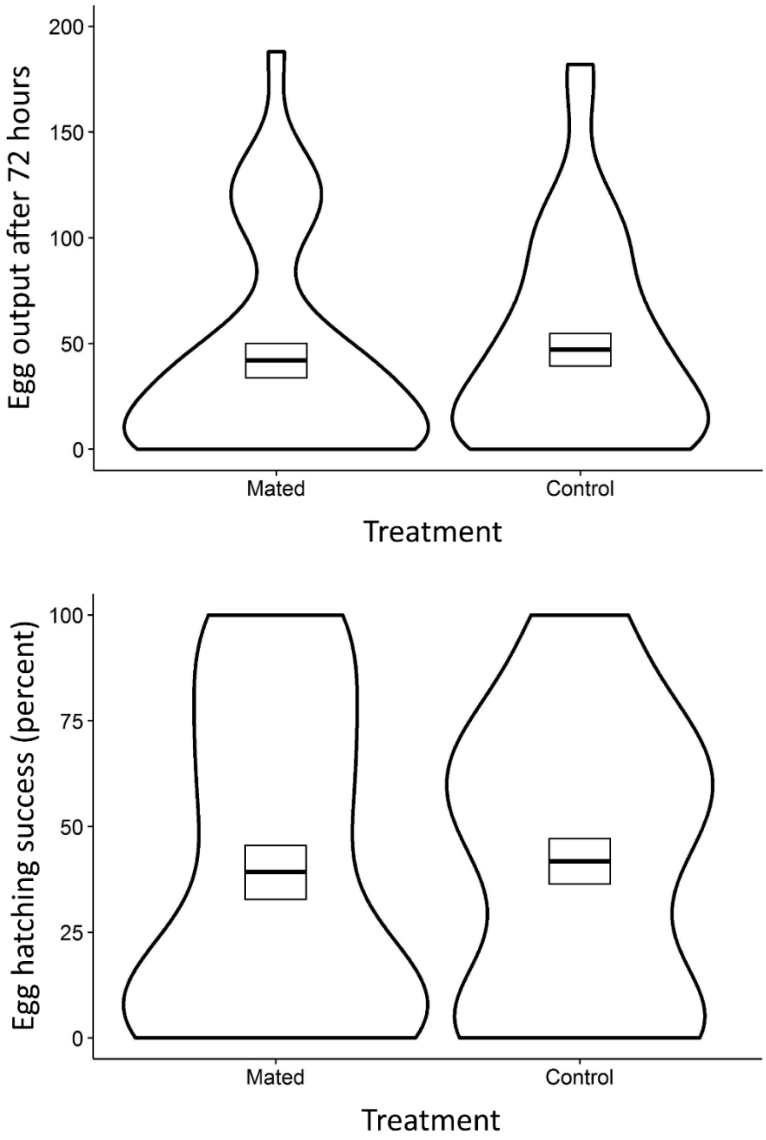


Figure 3. Violin plots showing the effects of the mated and control treatments on male fecundity in Experiment 2. Bars represent mean ± SE.

Discussion

Using the neriid fly *Telostylinus angusticollis*, we investigated whether frequent mating resulted in a condition-dependent depletion of ejaculate components that influence male fecundity and offspring traits that are likely to be related to fitness, and if males showed decreased interest in mating with novel, virgin females after a bout of frequent mating. In Experiment 1, we did not detect an effect of male mating history or male condition (larval nutrient availability) on male fecundity (female latency to lay eggs or egg hatching success) or offspring traits (egg-to-adult viability or offspring body size). Therefore, frequent mating

– regardless of male condition – did not deplete the ejaculate components required to induce oviposition, fertilise eggs, or confer non-genetic paternal effects to offspring viability or size. While there was no evidence that prior mating decreases male post-copulatory performance, we did find that previously mated males had a longer latency to mate with the single novel female compared to control (virgin) males, demonstrating that previously mated males were reluctant to re-mate immediately after a bout of frequent mating. Likewise, in Experiment 2, we found that males in the mated treatment group suffered a reduction in mating rate, and this reduction was not alleviated when exposed to multiple, novel females. Yet, we still found no effect of prior mating on fecundity across the pooled females. Thus, *T. angusticollis* males do not appear to suffer a substantial cost of mating on the ejaculate components required to fertilise eggs, induce oviposition, or affect traits related to offspring quality, but male prudence in mating may be mitigating any such costs.

Male neriid flies may be selected to invest equally in post-copulatory performance in each mating as females of this species are highly promiscuous and can store sperm from multiple males. Thus, males may be investing heavily in their ejaculate expenditure at a cost to their mating rate in order to be competitive during sperm competition. Such trade-offs between mating frequency and ejaculate expenditure have been suggested in the theoretical and empirical literature (Pitnick & Markow, 1994; Parker & Ball, 2005; Parker & Pizzari, 2010; Reinhardt *et al.*, 2011), and this trade-off can depend on the level of sperm competition (Parker and Pizzari, 2010). Males that transfer more sperm and seminal fluid products per mating are likely to be more competitive during sperm competition (e.g. Parker, 1990; Schärer *et al.*, 2004; Wigby *et al.*, 2009; Avila *et al.*, 2011; South and Lewis, 2011; Vellnow *et al.*, 2018). For example, males that have evolved under high sperm competition may be under strong selection to reduce mating number in order to invest equally in ejaculate transfer per mating. However, such strategies likely depend on the function of ejaculate allocation on fitness. For example, if transferring less than a certain amount of ejaculate results in zero fertilisations, then a strategy where males reduce mating rate to conserve ejaculate stores would be expected to evolve. Alternatively, we would expect males to prioritise mating rate over ejaculate

transfer if the effect of the quantity of ejaculate transferred on fitness saturates quickly. Therefore, the ecology and mating system of a species is likely to determine the evolution of male allocation to mating rate versus ejaculate transfer. However, it must also be noted that male fecundity was quantified in non-competitive environments as completing such measures of male fecundity under sperm competition is logically challenging in this study system and would require paternity analyses on the developing embryos. It is possible that costs of frequent mating may become more apparent under a competitive environment as previously mated males may be less successful at fertilising eggs. Therefore, further studies should aim to examine the effects of sperm depletion under competitive environments.

Given that many post-copulatory traits are expected to be condition-dependent (Macartney *et al.*, 2019), and a nutrient-poor larval diet has been shown to reduce several post-copulatory traits in *T. angusticollis,* as well as offspring body size (Bonduriansky & Head, 2007; Macartney *et al.*, 2018b), it is interesting that we did not detect any condition-dependent differences in male post-copulatory performance. This may be because males reared on the poor larval diet were observed mating fewer times while in the mated treatment compared to males reared on the rich larval diet, resulting in them conserving their smaller sperm and seminal fluid reserves. Additionally, male neriid flies transfer a very small ejaculate, estimated at ~1/10,000 of male body volume (Bonduriansky & Head, 2007), and this small ejaculate may enable males of both high and low condition to maintain sperm and seminal fluid stores. Furthermore, we did not detect any effect of larval diet on offspring body size after controlling for sire family, as shown previously in this species (Bonduriansky & Head, 2007; Crean *et al.*, 2014). The absence of such an effect may be due to differences in experimental design and the mechanism of transferring non-genetic factors in this species. A previous study on *T. angusticollis* has suggested that non-genetic effects on offspring are mediated by components in the seminal fluid rather than the sperm, meaning that the transfer of such factors is not tied to fertilisation (Crean *et al.,* 2014). Additionally, previous studies have paired males with newly emerged females instead of sexually mature females, and eggs may be more permeable to non-genetic factors in the seminal fluid prior to

sexual development. Therefore, further research into the mechanisms involved in the transfer of non-genetic factors is needed in this species.

Reproductive investment can also incur latent costs. A number of studies have shown that frequent mating is correlated with a reduction in longevity (Cordts & Partridge, 1996; Kotiaho & Simmons, 2003; McNamara, Elgar, & Jones, 2008; Perry & Tse, 2013), and may also affect reproductive aging (Koppik, Ruhmann, & Fricke, 2018). While we did not measure such latent costs in this study, it is possible that males that mate frequently may incur costs to lifespan. However, early life reproductive investment and short-term costs are likely to be more relevant as life expectancy is short in natural populations of this species (Kawasaki *et al.*, 2008). There is also evidence to suggest that males can incur costs due to the perception of females without directly mating (Gendron *et al.*, 2014; Harvanek *et al.*, 2017) which may mean that control males also experience some degree of cost – reducing our ability to detect differences in reproductive performance between mated and control males. However, such an explanation would not explain the differences in male mating rate when presented with novel females as males from the control treatment were more willing to mate with novel females compared to males from the mated treatment. Furthermore, in comparison to other insect species, including other Diptera, male neriid flies achieved a high number of matings without any detectable costs to post-copulatory performance (Hihara, 1981; Linklater *et al.*, 2007; Reinhardt *et al.*, 2011; Michaud *et al.*, 2013; Perry & Tse, 2013; Hopkins *et al.,* 2019). It is possible that further matings would cause males to suffer ejaculate depletion, but due to the clear reduction in mating rate, a higher number of matings is unlikely in the wild.

Conclusions

In summary, *T. angusticollis* males do not appear to suffer a cost of mating to fecundity or ability to produce good quality offspring, nor is there an effect of male condition whereby males reared on the nutrient poor larval diet incur a greater cost of mating compared to males reared on the nutrient rich larval diet. However, costs to post-copulatory performance appear to be mediated by changes in male mating behaviour: previously mated males reduced their

mating rate, even when provided with multiple novel females, and they also had a longer mating latency when paired with a single novel female. Therefore, *T. angusticollis* males may be selected to prioritise post-copulatory investment per mating over mating number, potentially due to the high risk of sperm competition in this species. In other words, if reducing ejaculate transfer per mating would result in a very low fertilisation probability, the best strategy could be to mate fewer times while investing fully in each mating. Future studies should further investigate condition-dependent ejaculate depletion, especially under sperm competition, as this would shed light on how males change reproductive investment strategies based on male condition and ability to invest in competing against other males.

References

ADLER, M.I. & BONDURIANSKY, R. (2013) Paternal effects on offspring fitness reflect father's social environment. *Evolutionary Biology* **40**, 288–292.

AVILA, F.W., SIROT, L.K., LAFLAMME, B.A., RUBINSTEIN, C.D. & WOLFNER, M.F. (2011) Insect seminal fluid proteins: identification and function. *Annual Review of Entomology* **56**, 21–40.

BATES, D., MAECHLER, M., BOLKER, B. & WALKER, S. (2015) lme4: Linear mixed-effects models using Eigen and S4. *Journal of Statistical Software* **67**, 1–48.

BONDURIANSKY, R. & CREAN, A.J. (2017) What are parental condition-transfer effects and how can they be detected? *Methods in Ecology and Evolution* **9**, 1–7.

BONDURIANSKY, R. & HEAD, M. (2007) Maternal and paternal condition effects on offspring phenotype in *Telostylinus angusticollis* (Diptera: Neriidae). *Journal of Evolutionary Biology* **20**, 2379–2388.

CORDTS, R. & PARTRIDGE, L. (1996) Courtship reduces longevity of male *Drosophila melanogaster*. *Animal Behaviour* **52**, 269–278.

CREAN, A.J. & BONDURIANSKY, R. (2014) Semenial fluid and mate choice: new predictions. *Trends in Ecology and Evolution* **31,** 253–255.

CREAN, A.J. & BONDURIANSKY, R. (2014) What is a paternal effect? *Trends in Ecology & Evolution* **29**, 554–559.

CREAN, A.J., KOPPS, A.M. & BONDURIANSKY, R. (2014) Revisiting telegony: offspring inherit an acquired characteristic of their mother's previous mate. *Ecology Letters* **17**, 1545–1552.

DELCURTO, H., WU, G. & SATTERFIELD, M.C. (2013) Nutrition and reproduction : links to epigenetics and metabolic syndrome in offspring. *Current opinion in clinical nutrition and metabolic care* **16**, 385–391.

DEWSBURY, D.A. (1982) Ejaculate cost and male choice. *American Naturalist* **119**, 601–610.

DEWSBURY, D.A.A. (1981) Effects of novelty on copulatory behavior: the Coolidge effect and related phenomena. *Psychological Bulletin* **89**, 464–482.

DRONEY, D.C. (1998) The influence of the nutritional content of the adult male diet on testis mass, body condition and courtship vigour in a Hawaiian Drosophila. *Functional Ecology* **12**, 920–928.

EVANS, J.P., LYMBERY, R.A., WIID, K.S., RAHMAN, M. & GASPARINI, C. (2017) Sperm as moderators of environmentally induced paternal effects in a livebearing fish. *Biology Letters* **13**, 10–13.

GARCIA-GONZALEZ, F. & SIMMONS, L.W. (2010) Male-induced costs of mating for females compensated by offspring viability benefits in an insect. *Journal of Evolutionary Biology* **23**, 2066–2075.

GENDRON, C.M., KUO, T.H., HARVANEK, Z.M., CHUNG, B.Y., YEW, J.Y., DIERICK, H.A. & PLETCHER, S.D. (2014) *Drosophila* life span and physiology are modulated by sexual perception and reward. *Science* **343**, 544–548.

HARVANEK, Z.M., LYU, Y., GENDRON, C.M., JOHNSON, J.C., KONDO, S., PROMISLOW, D.E.L. & PLETCHER, S.D. (2017) Perceptive costs of reproduction drive ageing and physiology in male *Drosophila*. *Nature Ecology and Evolution* **1**, 1–8.

HIHARA, F. (1981) Effects of the male accessory gland secretion on oviposition and remating in females of *Drosophila melanogaster*. *Zoology Magazine*. **90**, 307–316.

HOPKINS, B.R., SEPIL, I., THÉZÉNAS, M.-L., CRAIG, J.F., MILLER, T., CHARLES, P.D., FISCHER, R., KESSLER, B.M., BRETMAN, A., PIZZARI, T. & WIGBY, S. (2019) Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* **116**, 17925–17933.

KAWASAKI, N., BRASSIL, C.E., BROOKS, R.C. & BONDURIANSKY, R. (2008) Environmental effects on the expression of life span and aging: an extreme contrast between wild and captive cohorts of *Telostylinus angusticollis* (Diptera: Neriidae). *American Naturalist* **172**, 346–357.

KOPPIK, M., RUHMANN, H. & FRICKE, C. (2018) The effect of mating history on male reproductive ageing in *Drosophila melanogaster*. *Journal of Insect Physiology* **111**, 16–24.

KOTIAHO, J.S. & SIMMONS, L.W. (2003) Longevity cost of reproduction for males but no longevity cost of mating or courtship for females in the male-dimorphic dung beetle *Onthophagus binodis*. *Journal of Insect Physiology* **49**, 817–822.

KUZNETSOVA, A., BROCKHOFF, P. & RUNE, H. (2017) lmerTest package: tests

in linear mixed effects models. *Journal of Statistical Software* **82**, 1–26.

LINKLATER, J.R., WERTHEIM, B., WIGBY, S. & CHAPMAN, T. (2007) Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. *Evolution* **61**, 2027–2034.

MACARTNEY, E.L. (2015) Male reproductive performace: influence of age, sex and diet on fecundity and offspring quality. University of New South Wales. *MSc Thesis*.

MACARTNEY, E.L., CREAN, A.J. & BONDURIANSKY, R. (2017) Adult dietary protein has age- and context-dependent effects on male post-copulatory performance. *Journal of Evolutionary Biology* **38**, 42–49.

MACARTNEY, E.L., CREAN, A.J. & BONDURIANSKY, R. (2018a) Epigenetic paternal effects as costly, condition-dependent traits. *Heredity* **121**, 248–256.

MACARTNEY, E.L., CREAN, A.J., NAKAGAWA, S. & BONDURIANSKY, R. (2019) Effects of nutrient limitation on sperm and seminal fluid: a systematic review and meta-analysis. *Biological Reviews* **94**, 1722–1739.

MACARTNEY, E.L., NICOVICH, P.R., BONDURIANSKY, R. & CREAN, A.J. (2018b) Developmental diet irreversibly shapes male post-copulatory traits in the neriid fly *Telostylinus angusticollis*. *Journal of Evolutionary Biology* **31**, 1894–1902.

MARCOTTE, M., DELISLE, J. & MCNEIL, J.N. (2007) Effects of different male remating intervals on the reproductive success of *Choristoneura rosaceana* males and females. *Journal of Insect Physiology* **53**, 139–145.

MAYNARD SMITH, J. (1977) Parental investment: a prospective analysis. *Animal Behaviour* **25**, 1–9.

MCNAMARA, K.B., ELGAR, M.A. & JONES, T.M. (2008) A longevity cost of re-mating but no benefits of polyandry in the almond moth, *Cadra cautella*. *Behavioral Ecology and Sociobiology* **62**, 1433–1440.

MICHAUD, J.P., BISTA, M., MISHRA, G. & SINGH, O. (2013) Sexual activity diminishes male virility in two Coccinella species: consequences for female fertility and progeny development. *Bulletin of Entomological Research* **103**, 570–577.

MIRHOSSEINI, M.A., MICHAUD, J.P., JALALI, M.A. & ZIAADDINI, M. (2014) Paternal effects correlate with female reproductive stimulation in the

polyandrous ladybird *Cheilomenes sexmaculata*. *Bulletin of Entomological Research* **104**, 480–485.

MULLER, K., ARENAS, L., THIÉRY, D. & MOREAU, J. (2016) Direct benefits from choosing a virgin male in the European grapevine moth, *Lobesia botrana*. *Animal Behaviour* **114**, 165–172.

PARKER, G.A. (1990) Sperm competition games: raffles and roles. *Proceedings of the Royal Society B: Biological Sciences* **242**, 120–126.

PARKER, G.A. & BALL, M.A. (2005) Sperm competition, mating rate and the evolution of testis and ejaculate sizes: a population model. *Biology Letters* **1**, 235–238.

PARKER, G.A. & PIZZARI, T. (2010) Sperm competition and ejaculate economics. *Biological Reviews* **85**, 897–934.

PERRY, J.C. & TSE, C.T. (2013) Extreme costs of mating for male two-spot ladybird beetles. *PLoS ONE* **8**, 1–5.

PITNICK, S. & MARKOW, T.A. (1994) Male gametic strategies - sperm size, testis size, and the allocation of ejaculate among successive mates by the sperm-limited fly *Drosophila pachea* and its relatives. *American Naturalist* **143**, 785–819.

PIZZARI, T., CORNWALLIS, C.K., HANNE, L., JAKOBSSON, S. & BIRKHEAD, T.R. (2003) Sophisticated sperm allocation in male fowl. *Nature* **426**, 70–74.

POLAK, M., SIMMONS, L.W., BENOIT, J.B., RUOHONEN, K., SIMPSON, S.J. & SOLON-BIET, S.M. (2017) Nutritional geometry of paternal effects on embryo mortality. *Proceedings of the Royal Society B: Biological Sciences* **284**, 1–9.

PRESTON, B.T., STEVENSON, I.R., PEMBERTON, J.M. & WILSON, K. (2001) Dominant rams lose out by sperm depletion. *Nature* **409**, 681–682.

RANDO, O.J. (2012) Daddy Issues: Paternal effects on phenotype. *Cell* **151**, 702–708.

RANDO, O.J. (2016) Intergenerational transfer of epigenetic information in sperm. *Cold Spring Harbor Perspectives in Biology* **6**, 1–14.

RASBAND, W.S. (2015) ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA. *http://imagej.nih.gov/ij/*.

REINHARDT, K., NAYLOR, R. & SIVA-JOTHY, M.T. (2011) Male mating rate is constrained by seminal fluid availability in bedbugs, *Cimex lectularius*. *Plos*

One **6**, 1–8.

SCHÄRER, L., LADURNER, P. & RIEGER, R.M. (2004) Bigger testes do work more: experimental evidence that testis size reflects testicular cell proliferation activity in the marine invertebrate, the free-living flatworm *Macrostomum* sp. *Behavioral Ecology and Sociobiology* **56**, 420–425.

SENTINELLA, A., CREAN, A. & BONDURIANSKY, R. (2013) Dietary protein mediates a trade-off between larval survival and the development of male secondary sexual traits. *Functional Ecology* **27**, 1134–1144.

SCHAALJE, G.B., MCBRIDE, J.B. & FELLINGHAM, G.W. (2002) Adequacy of approximations to distributions of test statistics in complex mixed linear models. *Journal of Agricultural, Biological, and Environmental Statistics* **7**, 512–524.

SHELDON, B.C. (2002) Relating paternity to paternal care. *Philosophical Transactions of the Royal Society B: Biological Sciences* **357**, 341–350.

T. H CLUTTON-BROCK (1991) *The Evolution of Parental Care*. Princeton University Press. Princeton, USA.

TORRES-VILA, L.M. & JENNIONS, M.D. (2005) Male mating history and female fecundity in the Lepidoptera: do male virgins make better partners? *Behavioral Ecology and Sociobiology* **57**, 318–326.

VAHED, K. (1998) The function of nuptial feeding in insects: review of empirical studies. *Biological Reviews of the Cambridge Philosophical Society* **73**, 43–78.

VELLNOW, N., MARIE-ORLEACH, L., ZADESENETS, K.S. & SCHÄRER, L. (2018) Bigger testes increase paternity in a simultaneous hermaphrodite, independently of the sperm competition level. *Journal of Evolutionary Biology* **31**, 180–196.

WANG, Y., LIU, H. & SUN, Z. (2017) Lamarck rises from his grave : parental environment-induced epigenetic inheritance in model organisms and humans. *Biological Reviews* **92**, 2084–2111.

WIGBY, S., SIROT, L.K., LINKLATER, J.R., BUEHNER, N., CALBOLI, F.C.F., BRETMAN, A., WOLFNER, M.F. & CHAPMAN, T. (2009) Seminal fluid protein allocation and male reproductive success. *Current Biology* **19**, 751–757.

ZAJITSCHEK, F., ZAJITSCHEK, S. & MANIER, M. (2017) High-protein paternal

diet confers an advantage to sons in sperm competition. *Biology Letters* **13**, 1–5.

Chapter Six

THE COMPLEX CONDITION DEPENDENCE OF MALE REPRODUCTIVE PERFORMANCE IN *DROSOPHILA MELANOGASTER*

Erin L Macartney, Valérian Zeender, Abhishek Meena, Russell Bonduriansky, Stefan Lüpold

ELM conceived the project, designed the experiment with the help of VZ and SL, ran the experiment with the help of VZ, AM and SL. ELM analysed the data and wrote the manuscript. RB and SL provided valuable advice throughout all phases of the manuscript.

Abstract

Sexual traits, including post-copulatory traits, can respond strongly to environmental factors such as nutrient availability, and can have high levels of additive genetic variation. Environmental factors can alter male condition and ability to invest in sexual traits, and male genotype can interact with the environment to alter sexual trait expression. Furthermore, ejaculate components can be rapidly depleted across successive matings due to the cost of, and limitations in, sperm and seminal fluid synthesis. Yet, little is known about how nutrition and genotype affect male ability to mate multiply and maintain ejaculate allocation across successive matings. Using *Drosophila melanogaster* that had been genetically engineered to express green fluorescent sperm, we reared larvae on high, intermediate, and low nutrient concentrations across 17 independent isoline crosses. As adults, we then presented males with five mating opportunities within 8hrs, and measured mating behaviour and the number of sperm transferred at each mating. This allowed us to test for both nutritional and genetic effects on male reproductive performance. We found that mating latency increased and the number of sperm transferred decreased with successive matings. Diet and isoline both independently affected mean sperm number, but not sperm depletion rate. Males that mated more often during the mating assay (high 'mating rate') also had shorter mating latencies and mating durations, and transferred more sperm across matings, meaning that some males, across all diets and isolines, were better able to invest in both mating rate and sperm transfer. However, mating rate was not affected by diet or isoline, suggesting that an unknown factor accounted for a substantial proportion of variation in male copulatory performance. We hypothesize that this factor could be stochastic developmental variation. Our findings raise important questions about sources of variation in male condition and reproductive investment.

Introduction

Sexual traits important for fitness are often subject to persistent directional selection, yet such traits are highly variable in their expression (Andersson, 1994). Often, this variation is due to high environmental plasticity; traits that have been subject to persistent directional selection are costly to produce and maintain, and environmental factors such as nutrient availability or stress can alter male ability to invest in costly traits. For example, males that experience a low nutrient diet have fewer metabolic resources (i.e., are in low phenotypic condition) and are less able to invest in costly sexual traits important for male fitness (i.e., trait expression is condition-dependent) (Andersson, 1982; Nur & Hasson, 1984; Grafen, 1990; Iwasa, Pomiankowski, & Nee, 1991). Additionally, sexual traits generally have high levels of additive genetic variation compared to nonsexual ("metric") traits (Houle, 1992; Pomiankowski & Moller, 1995; Ward, 2000; Simmons & Kotiaho, 2002), and genetic effects can interact with environmental effects to alter trait responses to the environment (Rowe & Houle, 1996; Hunt *et al.*, 2004; Hill, 2011). For example, certain genotypes may be better able to overcome environmental stressors such as nutrient limitation, thus reducing the strength of condition-dependent trait responses due to diet within certain genotypes. Therefore, diet can influence male condition by directly altering the metabolic resource pool available for investment in costly sexual traits, but sexual traits can also depend on additive genetic variation, and the strength of condition-dependent responses to environmental variation can be genotype-dependent (e.g., Gienapp & Merilä, 2010; Ingleby, Hunt, & Hosken, 2010).

Effects of environment and genotype are not only important for pre-copulatory signalling traits, but also for post-copulatory traits that are costly and important for male fitness (Ward, 2000; Simmons & Kotiaho, 2002; Perry & Rowe, 2010; Evans, Rahman, & Gasparini, 2015; Kahrl & Cox, 2015; Macartney *et al.*, 2019). Post-copulatory traits can show condition-dependent trait expression in response to variation in nutrient availability (reviewed in Macartney *et al.,* 2019), but few studies have examined dietary and genetic effects concurrently on post-copulatory trait expression (but see Evans *et al.*, (2015) for diet and genotypic effects on sperm velocity, sperm length and

number in the guppy *Poecilia reticulata*). Under 'raffle' sperm competition, sperm number is a highly important trait that can determine the outcome of sperm competition (Parker, 1970, 1990; Gage & Morrow, 2003), and a recent meta-analysis found that sperm number is more strongly reduced under nutrient limitation compared to sperm quality (Macartney *et al.*, 2019). However, many studies examining differences in sperm number measure the number of sperm produced/stored rather than the number of sperm transferred at mating (e.g., Rahman *et al.*, 2013; Melo *et al.*, 2014; O'Dea *et al.*, 2014; Kaldun and Otti, 2016; but see Engqvist, 2008; Vermeulen *et al.*, 2008; Perry and Rowe, 2010; Costa *et al.*, 2012). While this measure is informative of male potential to invest in sperm transfer, it does not provide information on male post-copulatory investment strategies. For example, males of low condition may have a reduced ability to produce large numbers of sperm, but if low-condition males rarely achieve mating, such males may strategically allocate relatively more sperm to a single mating compared to high-condition males (e.g., Rowe and Arnqvist, 1996; Danielsson, 2001; Fricke *et al.*, 2015). Such conditional tactics in post-copulatory reproductive investment have been demonstrated in the ladybird, *Adalia bipunctata*, where low-condition males that had limited access to food transferred relatively more sperm compared to high-condition males, but the latter transferred larger spermatophores comprised of more non-sperm components (Perry & Rowe, 2010). While competitive fertilisation success is not solely determined by sperm number (see Lüpold et al., 2012), sperm number can be used as an indicator of strategic ejaculate investment under raffle competition.

Males of many species suffer sperm depletion across successive matings (Dewsbury, 1982; Torres-Vila & Jennions, 2005; Linklater *et al.*, 2007; Muller *et al.*, 2016). The rate of depletion is also likely to depend on both the ability of males to invest in ejaculate transfer (i.e., condition) and their investment in previous matings (e.g., Hopkins *et al.*, 2019). For example, low- and high-condition individuals may invest differently in initial matings, and this initial investment could influence their investment in subsequent matings: males that invest heavily in initial matings could suffer more rapid sperm depletion when presented with additional mating opportunities. Alternatively, males may adjust

their mating behaviour to mitigate ejaculate depletion, as observed in the neriid fly, *Telostylinus angusticollis* (Macartney *et al.* Chapter Five). Yet, to date, no studies have examined male investment in sperm transfer across multiple matings as a function of two important condition-determining factors – nutrient availability and genotype. Assessing dietary and genetic effects on sperm transfer across multiple matings will provide us with a more thorough understanding of condition-dependent investment in mating and how such investment can change across successive mating events. Such differences in male ability to invest in ejaculate transfer across matings are likely to have important consequences for male fitness under sperm competition (see Hopkins *et al.*, 2019).

Drosophila species have extraordinarily large sperm, which is likely to result in a high cost of sperm production (Lüpold *et al.*, 2016). Additionally, the number of sperm transferred at mating can be influenced by the availability of protein during development in *D. melanogaster* (McGraw *et al.*, 2007), and sperm depletion can occur rapidly in this species (e.g., Linklater *et al.*, 2007). However, it is not known whether nutrient availability in the larval diet affects the rate of sperm depletion experienced by males across successive matings, or whether these effects are genotype-dependent. To address these questions, we tested the effects of nutrient concentration in the larval diet (as a manipulation of male condition) across 17 independent isolines (used to test for genetic effects) on male mating behaviour and sperm transfer across successive matings in *Drosophila melanogaster*. To facilitate the quantification of sperm transferred at mating, we used flies that had been genetically engineered to express green fluorescent protein in their sperm heads (Manier *et al.*, 2010; Lüpold *et al.*, 2012). We predicted that larval diet would affect the mating behaviour (mating latency and mating duration) and sperm transfer of the adult males. Specifically, we predicted that males reared on a diet with a lower nutrient concentration (i.e., low-condition males) would transfer fewer sperm and experience a steeper rate of sperm depletion compared to males reared on a high-nutrient diet (i.e., high-condition males). This prediction was based on the assumption that low-condition males would incur relatively higher costs of sperm production, have smaller total sperm reserves and might invest relatively

more in earlier matings if they anticipate fewer mating opportunities in the future. We also predicted that isolines would vary in male mating behaviour and sperm transfer (i.e., these traits would exhibit broad-sense genetic variation; e.g., see Lüpold *et al.*, 2012), and that effects of isoline and diet would interact (reflecting a genotype-by-environment interaction).

Materials and Methods

Study animals

All experimental *Drosophila melanogaster* came from pairwise crosses of independent isogenic lines (i.e., approximate 'clones'), originally derived from a large LH_m stock population. These lines are expected to have a homozygosity of 96% after 15 generations of full-sibling inbreeding (Falconer & Mackay, 1996). Crosses between males of one clonal population and virgin females from another were used to generate heterozygous, but still quasi-clonal individuals (henceforth "isolines"). Using these individuals enabled us to subject the same genotypes to different treatments simultaneously, replicated across multiple individuals per genotype. 17 independent isolines were used to generate focal males. All males were genetically engineered to express green-fluorescent protein (GFP) in their sperm heads, thus facilitating the quantification of their sperm transferred to a female (Manier *et al.*, 2010). All females used in the experiments were derived from a single cross of two non-GFP isogenic lines.

Larvae from each of the 17 isolines were reared on either a 'high', 'intermediate' or 'low' nutrient diet to create adult males of high, intermediate and low condition within each isoline. We predicted that we would detect a 'gradient' of condition-dependent responses between males of high, intermediate, and low condition (i.e., males from the intermediate diet would express 'intermediate' trait responses compared to males from the high and low diets). The high diet consisted of 12.5ml cornmeal medium (75 g glucose, 100 g fresh yeast, 55 g maize, 80 g agar, 100 g flour, 10 ml Nipagin antimicrobial agent per litre of food medium). The intermediate diet had 1/3 the nutrient concentration of the high diet, and the low diet had 1/9 the nutrient concentration of the high diet, diluted in water and agar to equal

consistency. All larvae were collected during the 1st instar and reared at equal density of 40 larvae per vial, replicated across 4 vials for each diet × isoline combination. Females were reared in culture bottles at moderate density. All vials and bottles were maintained at 24°C, 60% humidity, and a 14:10 light:dark cycle.

All experimental flies were collected within 8 hours of adult emergence and transferred in groups of 15 individuals to single-sex vials containing standard food medium, separated by treatment, isoline, and day of eclosion.

Sperm-depletion assay

At six days old, ten males per diet and isoline combination (i.e., 10 males x 3 diets x 17 isolines = 510 males in total) were used for the mating assays. Assays were conducted over two consecutive days, with replicates 1-5 conducted on the first day, and replicates 6-10 conducted the following day. For each block, five males were randomly selected from the pool of males from the same diet and isoline that had eclosed on the same day (in order to standardise age).

In order to avoid potential virgin effects where males normally perform worse on the first mating (see Bjork *et al.*, 2007), all experimental males were allowed to mate once with a standardised virgin female (standardised for age, diet, and genetic background) 24hrs prior to the sperm depletion assay (this was considered long enough to allow some replenishment of any depleted sperm). Males were observed until mating, then separated and housed individually until the mating assay the following day.

On the day of the mating assay, males were given the opportunity to mate sequentially with 5 different females over 8 hours. Each focal male was placed in a food vial with two standardised virgin females (also standardised for age, diet, and genetic background) and observed until mating commenced with one of the females. Immediately after mating initiation, the unmated female was removed by gentle aspiration without disturbing the mating pair. Immediately after copulation ended, the mated female was removed, and the focal male was provided with two new standardised virgin females. Mating latency (i.e., the

time between the previous mating and the new mating, or since entering the mating assay for the first mating) and mating duration were recorded for each mating. Females were frozen individually within 15 minutes of the end of mating to avoid sperm ejection (Manier et al., 2010; Lüpold et al., 2013), and males were frozen after achieving 5 matings or at the end of the 8-hr mating window.

To quantify the number of sperm transferred by males, the female reproductive tract was removed from the abdomen and placed into a drop of water on a microscope slide, the seminal receptacle was uncoiled with a fine probe, and a coverslip was placed over the sample and sealed with rubber cement. All sperm within the bursa, spermathecae, and seminal receptacle were counted under an Olympus BX51 fluorescence microscope (Olympus America, Melville, USA) with a green fluorescent filter, and summed to obtain the total number of sperm transferred.

Many males did not mate multiply, substantially reducing replication at later matings. In order to ensure adequate sample sizes within each diet and isoline combination, sperm were counted from the 8 isolines for which at least four males from each diet had obtained a minimum of three successful copulations. Subsequently, sperm were counted from all males that completed four or five matings. For logistical reasons, and because prior experience suggested a minimal decline in sperm numbers between the first and second mating, sperm were not counted from the second mating. All sperm counts were done blind to diet and isoline.

For all males whose sperm were quantified, thorax length was measured under a Leica MS5 stereo-microscope (Leica Microsystems, Heerbrugg, Switzerland) at 40 x magnification.

Statistical analysis

All analyses were completed in the statistical software package R version 3.3.2 (R Development Core Team, 2016), using the *lme4* (Bates *et al.*, 2015) and *lmerTest* (Kuznetsova, Brockhoff, & Rune, 2017) packages, with *p*-values for

Gaussian mixed effects models based on the Satterthwaite approximation for denominator degrees of freedom (Schaalje, McBride, & Fellingham, 2002).

Treatment effects on male thorax length were analysed using a general linear mixed-effects model with diet as the fixed effect and isoline as the random effect. The model also initially included a random interaction of diet and isoline, but it was not statistically significant and its removal improved the model fit (see Results).

Focal males varied considerably in the number of times they mated during the 8-hour assay (hereafter 'mating rate'). To investigate the factors contributing to variation in mating rate, mating rate was analysed using generalised linear mixed-effects models with a Poisson distribution, diet as a fixed effect, and isoline and observation (to correct for overdispersion) as random effects. The model also initially included a random interaction of diet and isoline, but its inclusion in the model did not improve model fit so was later removed (see Results). Male thorax length (mean-centred within diet treatment to eliminate co-linearity with the categorical effect of diet) was also included as a co-variate to determine whether variation in male thorax length within larval diets affected the total number of times the male mated. Thorax length was measured only for males in which sperm transfer was quantified (i.e., males that mated a minimum of three times). To account for potential covariation between mating rate, mating latency, mating duration, and sperm transfer, mating rate was also included as a main effect and as an interaction with diet in models of mating latency, mating duration and sperm transfer (diet x mating rate was later removed from all models as it did not improve model fit.

Differences in male mating latency (log-transformed to improve normality), mating duration and the number of sperm transferred at mating were analysed in general linear mixed-effects models with isoline and male ID as random effects. All models included main effects of diet, mating order (i.e., order of consecutive matings), mating rate (i.e., the number of matings during the assay period) and a diet x mating order interaction. Initially, models tested for linear and quadratic effects of mating order by including diet x mating order^2 and diet x centred mating order (mean-centred to avoid co-linearity with mating order^2),

but the quadratic term was later removed as it did not improve model fit. Models also initially included a random-effect interaction of diet x isoline, but this interaction also did not improve model fit (see Results) and so was removed from the final models. Mating duration was also initially included as a co-variate in sperm transfer analyses to test for correlations between mating duration and the number of sperm transferred, but this did not improve model fit so was removed from the model.

The significance of all interactions and co-variates was tested by likelihood ratio tests (LRTs) and *p*-values were obtained using the *car* package (Fox & Weisberg, 2019). Co-variate interactions and random interactions were removed if they did not improve model fit based on a conservative cut-off of LRT $p > 0.1$. Models were reduced in order to increase power of detecting main effects. The contribution of isoline to the total variance in the data was also calculated, and its contribution to model fit was investigated using LRTs.

Post-hoc comparisons between effects of diets on male thorax length and sperm transfer were conducted using the package *emmeans* and the Tukey method of significance (Lenth *et al.*, 2019).

Differences in variance between diets and isolines for all response variables were conducted using a Levene's test and the *car* package (Fox *et al.*, 2014).

Results

Male thorax length

Diet significantly affected male body size (estimate = -0.027, $t_{113} = -6.225$, $p < 0.001$), with the low diet resulting in a significantly smaller thorax compared to the intermediate and high diets. There was no difference in thorax length between males from the intermediate and high diets (Fig. 1). Isoline accounted for 10.84% of the total variance in body size and significantly improved model fit ($\chi^2_1 = 5.248$, $p = 0.022$), but the random interaction of diet and isoline did not improve model fit ($\chi^2_1 = 0.664$, $p = 0.717$). Therefore, both diet and isoline are independently important for male body size, but isoline does not significantly interact with diet to affect male body size.

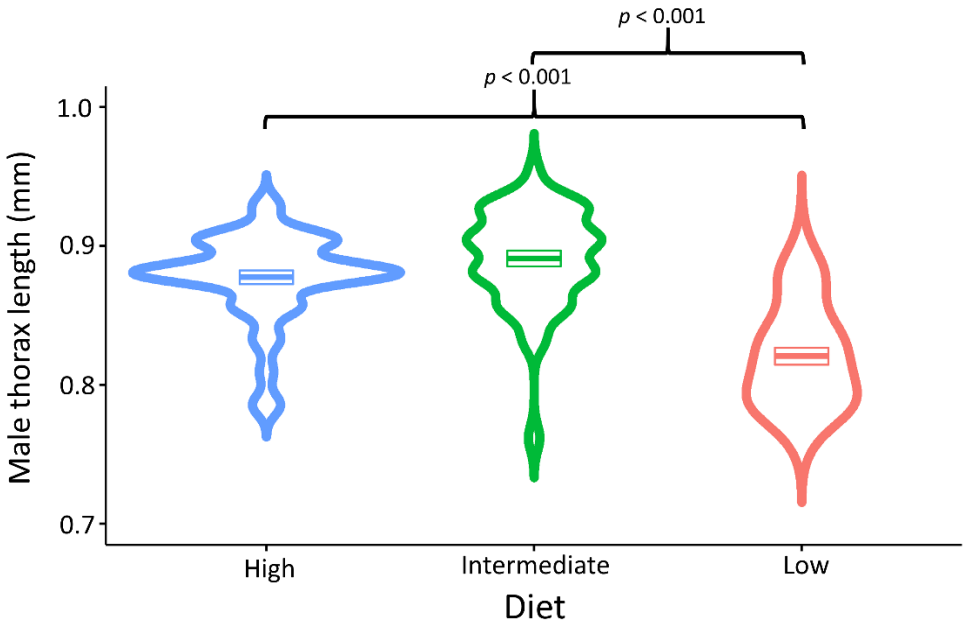


Figure 1. Effects of diet on male thorax length. Boxes show mean ± SE. Significant pair-wise comparisons are displayed above plots. Blue = high diet, green = medium diet, red = low diet.

Male mating behaviour and sperm depletion

The total number of matings achieved during the 8-hour assay period ('mating rate') varied among individual males from zero to five matings, with 36 out of the 510 males not mating during the assay period. However, mating rate was not affected by diet ($Z_{498} = 0.217$, estimate = 0.007, $p = 0.828$) (Fig. 2), and the inclusion of male thorax length (mean-centred within diet) did not improve model fit ($\chi^2_1 = 0.575$, $p = 0.448$). Similarly, isoline accounted for a negligible amount of the total variance in mating rate (isoline variance component <0.001%) and did not improve model fit ($\chi^2_1 = 0$, $p = 1$) (Fig. 3), nor was the interaction of diet and isoline important for model fit ($\chi^2_1 = 0.035$, $p = 0.983$). Therefore, mating rate was not strongly related to either diet during larval development or isoline, and was not affected by body size (after correcting for effects of diet) among males that mated three or more times.

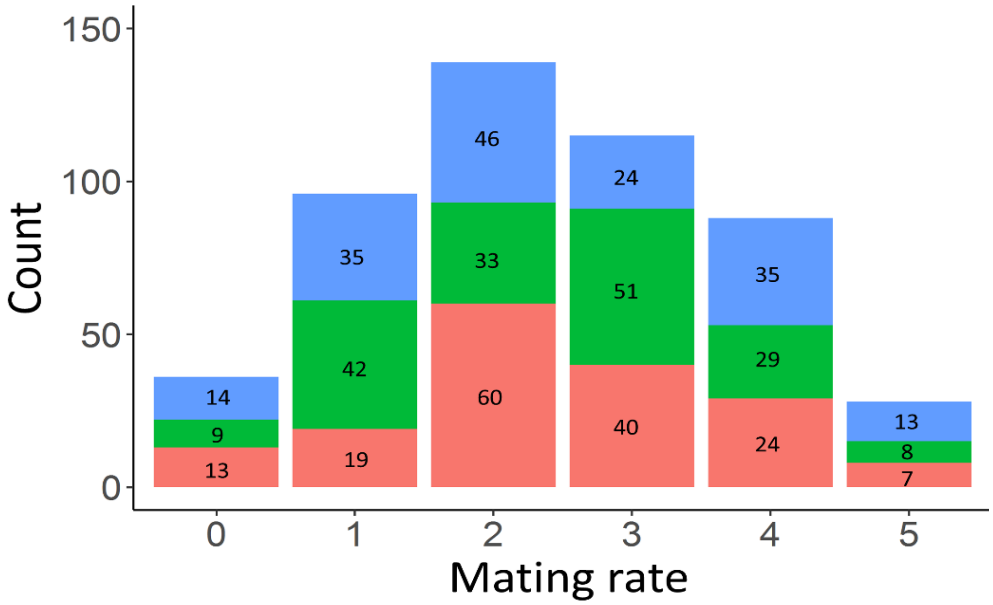


Figure 2. Frequency distribution of the total number of matings achieved by focal males during the 8-hour treatment period ('mating rate'). Blue = high diet, green = intermediate diet, red = low diet. Numbers represent the sample size (number of focal males) within each diet.

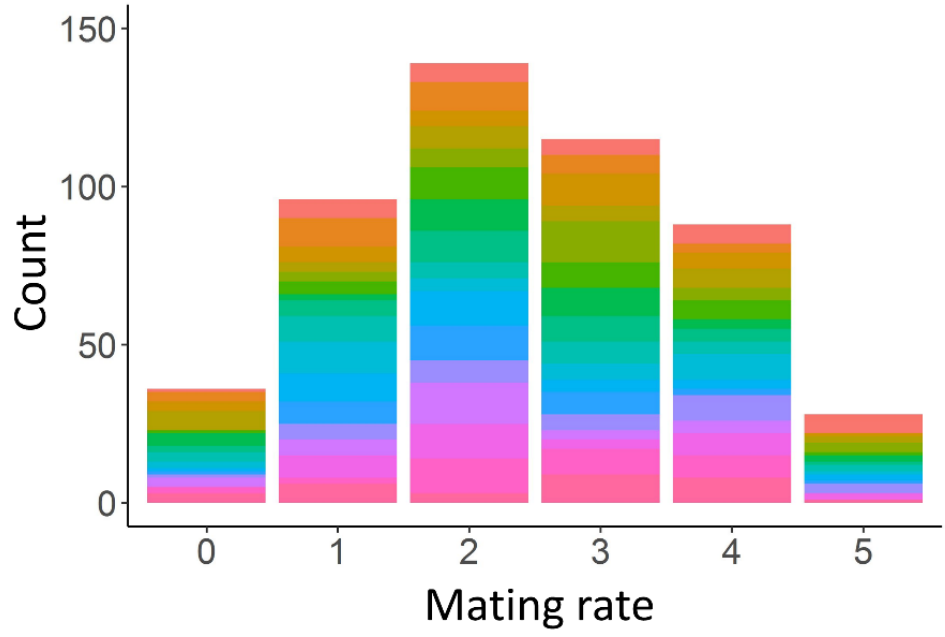


Figure 3. Frequency distribution of the total number of matings achieved by focal males during the 8-hour treatment period ('mating rate') with each colour representing one of the 17 isolines.

Mating latency, mating duration, and the number of sperm transferred were significantly correlated with mating rate (Table 1), in that males mating more often exhibited shorter mating latencies and shorter mating durations, but transferred significantly more sperm (Fig. 4a; 5a; 6a). This pattern was consistent across all diets (i.e., the diet x mating rate interaction was non-significant for all response variables) (Fig. 4bcd, 5bcd, 6bcd). Mating latency increased with each successive mating ('mating order'), but there was no effect of diet and no diet x mating order interaction (Table 1; Fig. 4; Fig. 7a), meaning that increase in mating latency was not influenced by diet. Isoline accounted for < 1% of the total variance in mating latency and did not improve the model fit ($\chi^2_1 = 1.259$, $p = 0.262$), nor did the inclusion of a diet x isoline random interaction improve model fit ($\chi^2_1 = 0.007$, $p = 0.996$).

We detected a near-significant diet x mating order interaction for mating duration, and a significant effect of diet on mating duration, with males from the low diet mating for longer (Table 1; Fig. 5, Fig. 7b). However, post-hoc pairwise comparisons did not detect any significant differences in mating duration between the diets (mating duration $_{\text{high-low comparison}}$: estimate = -0.960, $p = 0.232$; mating duration $_{\text{medium-low comparison}}$: estimate = -0.714, $p = 0.442$; mating duration $_{\text{high-medium comparison}}$: estimate = -0.247, $p = 0.912$). Isoline accounted for 5.56% of total variance for mating duration, and improved model fit ($\chi^2_1 = 25.478$, $p < 0.001$), but the inclusion of a diet x isoline random interaction did not improve model fit ($\chi^2_1 = 0.542$, $p = 0.463$). Therefore, diet and isoline independently affected mating duration, but isoline did not alter effects of diet.

Sperm transfer decreased with mating order, and males from the low diet transferred significantly fewer sperm overall compared to males from the intermediate and high diets (Table 1) (sperm transfer $_{\text{high-low comparison}}$: estimate = 111.5, $p = 0.043$; sperm transfer $_{\text{medium-low comparison}}$: estimate = 130.0, $p = 0.016$; sperm transfer $_{\text{high-medium comparison}}$: estimate = -18.4, $p = 0.914$). However, there was no diet x mating order interaction, meaning that the rate of sperm depletion was not affected by diet (Table 1; Fig. 6; Fig. 7c). Isoline accounted for 8.61% of the total variation in the number of sperm transferred, and was important for model fit ($\chi^2_1 = 4.761$, $p = 0.029$), but the inclusion of a diet x

isoline random interaction did not improve model fit ($\chi^2_1 = 1.108, p = 0.575$), meaning that isoline and diet were important for sperm transfer, but isoline did not mediate any effects of diet on sperm transfer.

Diet did not significantly affect the variance in male body size (Diet: $F_2 = 1.711, p = 0.187$), mating rate ($F_2 = 1.956, p = 0.142$), mating latency ($F_2 = 2.527, p = 0.080$), mating duration ($F_2 = 1.4588, p = 0.233$), or sperm transfer (Diet: $F_2 = 0.341, p = 0.711$). Isoline did not significantly affect the variance of male body size ($F_8 = 1.398, p = 0.205$), mating rate ($F_{16} = 1.382, p = 0.145$) or sperm transfer ($F_{16} = 0.726, p = 0.767$), but did significantly affect mating latency ($F_{16} = 1.794, p = 0.027$) and mating duration ($F_{16} = 1.791, p = 0.027$).

Table 1. Main and interactive effects of diet and mating order on male mating latency, mating duration, and sperm transfer, with mating rate as a covariate. Bold values indicate a significance value of $p < 0.05$. Negative effect sizes represent a decrease in the response variables with increased mating number, mating rate, or with decreased nutrients.

	Mating latency		Mating duration		Sperm transferred	
	Estimate	*p* value	Estimate	*p* value	Estimate	*p* value
Intercept	**5.289**	**<0.001**	**20.089**	**<0.001**	120.404	0.333
Diet	0.027	0.505	**1.438**	**0.014**	**-57.016**	**0.015**
Mating order	**0.178**	**0.003**	0.941	0.089	**-271.670**	**<0.001**
Mating rate	**-0.296**	**<0.001**	**-0.720**	**<0.003**	**130.970**	**<0.001**
Diet x mating order	0.024	0.396	-0.482	0.058	-7.285	0.613

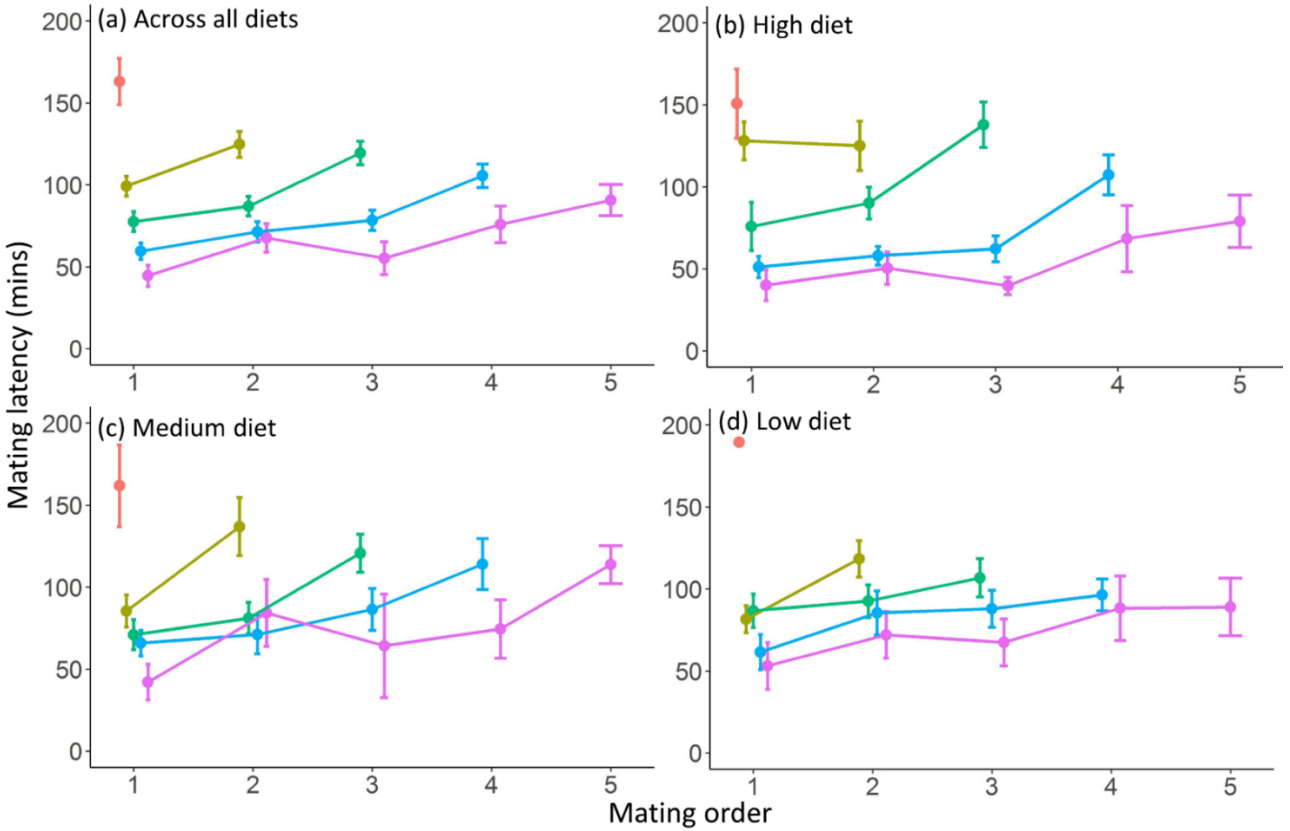


Figure 4. Effects of mating rate (i.e., the number of matings during the assay) (red = 1, mustard = 2, green = 3, blue = 4, pink = 5) and mating order (i.e., the order of consecutive matings) on mating latency across all diets (a), the high diet (b), the intermediate diet (c), and the low diet (d). Plots represent observed mean ± SE.

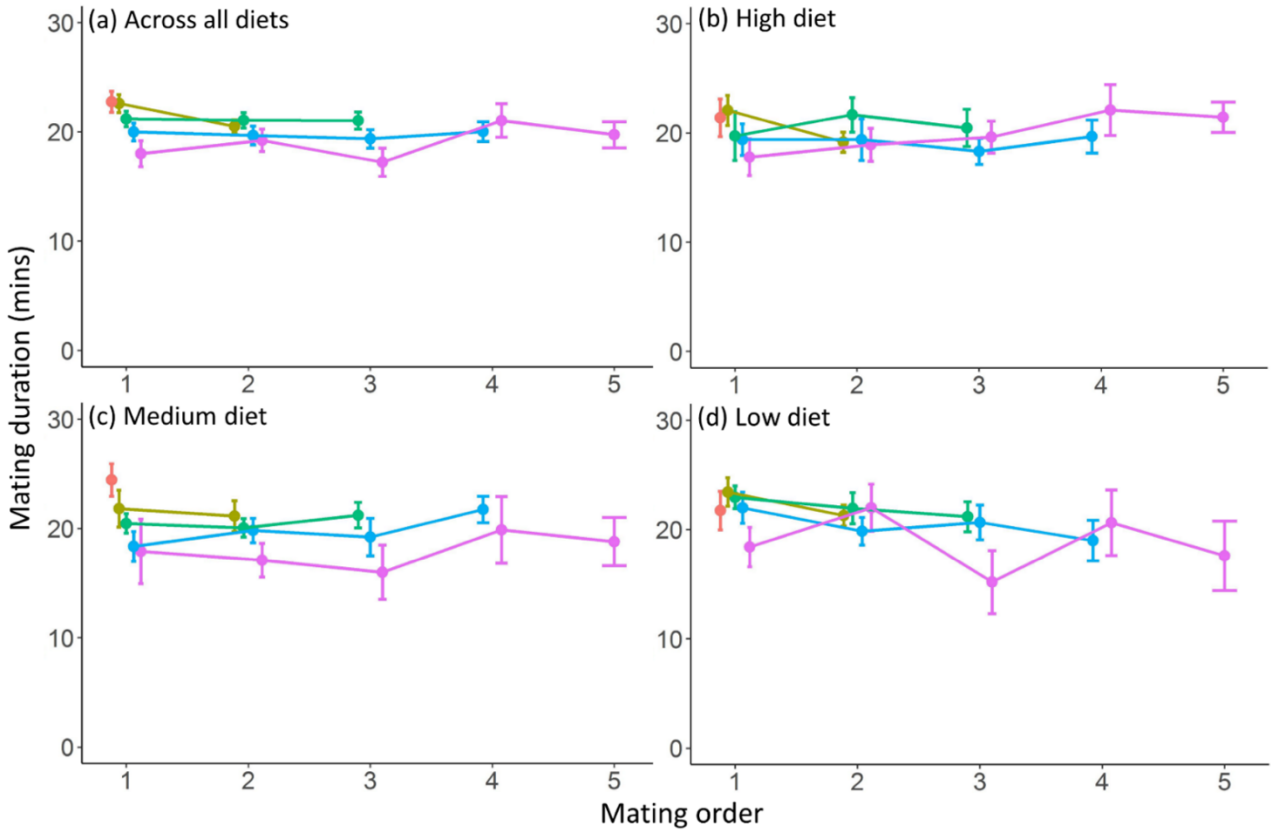


Figure 5. Effects of mating rate (i.e., the number of matings during the assay) (red = 1, mustard = 2, green = 3, blue = 4, pink = 5) and mating order (i.e., the order of consecutive matings) on mating duration across all diets (a), the high diet (b), the intermediate diet (c), and the low diet (d). Plots represent observed mean ± SE.

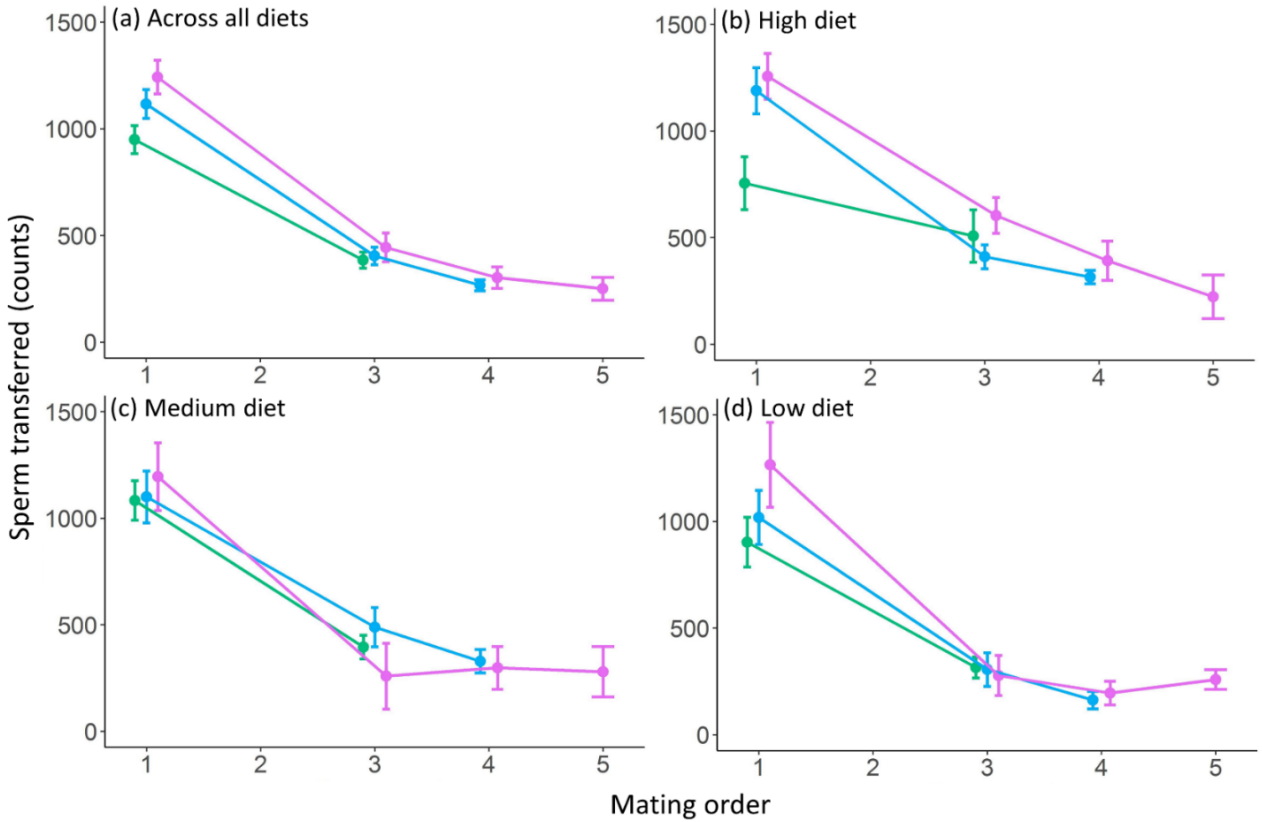


Figure 6. Effects of mating rate (i.e., the number of matings during the assay) (green = 3, blue = 4, pink = 5) and mating order (i.e., the order of consecutive matings) on sperm transferred across all diets (a), the high diet (b), the intermediate diet (c), and the low diet (d). Plots represent observed mean ± SE. Sperm were counted from males that mated a minimum of three times, and sperm from the 2nd mating were not counted).

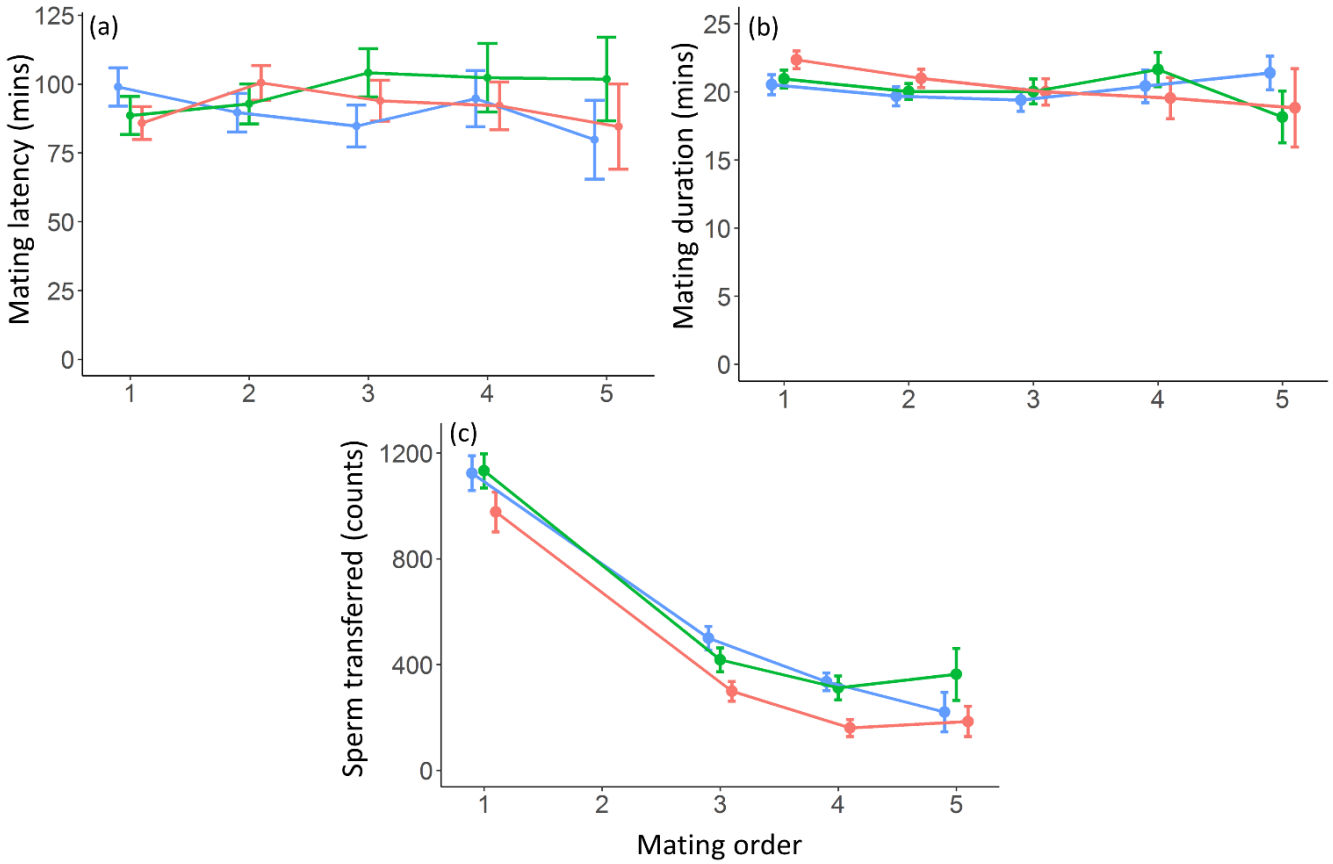


Figure 7. Effects of diet (blue = high diet, green = intermediate diet, red = low diet) and mating order on mating latency (a), mating duration (b), and sperm transferred (c). Plots represent observed mean ± SE.

Discussion

Here, we showed that males suffered sperm depletion over successive matings, manifested as a reduction in the number of sperm transferred with each additional mating. We also showed that mating latency increased with successive matings, suggesting that males require a longer refractory period to mate with additional females. These results suggest that mating is costly for males, whereby males are unable to maintain constant reproductive investment when provided with multiple mating opportunities over an 8-hour period. Additionally, we showed that males from the low nutrient larval diet transferred fewer sperm and mated for longer compared to males from the intermediate and high diets. The clear depletion of sperm with successive matings is consistent with other studies (Dewsbury, 1982; Preston *et al.*, 2001; Muller *et al.*, 2016), and the reduction in sperm transfer in males from the low diet is consistent with studies that have reported condition-dependent expression of sperm quantity (generally the amount of sperm stored/produced) in response to nutrient availability, especially during development in arthropods (reviewed in Macartney *et al.,* 2019; also see Macartney *et al.*, 2018). A longer mating duration in males from a low-nutrient diet is also consistent with other studies (e.g., Danielsson, 2001; Fricke *et al.*, 2015). Yet, surprisingly, the rate of sperm depletion and increase in mating latency with successive matings did not differ between diets, meaning that males from all diets likely invested the same proportion of total sperm across matings relative to their sperm stores. Furthermore, while isoline was important for model fit for sperm transfer and mating duration – indicating that these traits are partially genetically determined – the random interaction of diet and isoline was not important for mating latency, mating duration, or sperm transfer, suggesting that genetic background does not significantly alter condition-dependent effects of diet on male reproductive performance in *Drosophila melanogaster*. This contrasts with theoretical and empirical research that shows that male genotype can mediate sexual trait responses to environment (e.g., Rowe and Houle, 1996; Hunt *et al.*, 2004; Gienapp and Merilä, 2010; Ingleby *et al.*, 2010; Evans *et al.*, 2015).

Interestingly, males varied in the total number of matings they achieved during the assay period ('mating rate'), but there was no effect of diet or isoline on mating rate. Moreover, mating rate was strongly correlated with mating latency, mating duration, and sperm transfer: males that mated more often had shorter mating latencies and mating durations, and transferred more sperm. These patterns demonstrate a much higher propensity to mate and ability to transfer sperm in some males compared to others. Such clear differences in male mating rate and sperm transfer, independent of diet and isoline, suggest that some unmeasured factor might have a substantial influence on male reproductive investment.

Theoretical and empirical research suggests that there should be a trade-off between mating rate and ejaculate transfer, whereby males that invest heavily in mating rate should be prudent with sperm allocation (reviewed in Wedell, Gage, & Parker, 2002). Yet, we show that some males can invest in a relatively higher mating rate while also transferring more sperm compared to other males from the same diets and isolines. This suggests that these males are better able to invest in diverse aspects of reproduction. However, it is also possible that the males that had a lower mating rate and transferred fewer sperm were investing in a different reproductive strategy. For example, a low mating rate may reduce latent costs to other life-history traits such as lifespan, or a longer mating duration in these males may result from transferring more non-sperm components such as seminal fluid proteins. We did not find any evidence that mating duration is correlated with sperm transfer – consistent with other studies on *D. melanogaster* (see Lüpold *et al.*, 2011). Instead, mating duration may be correlated primarily with the transfer of non-sperm components (such as seminal fluid proteins) that can alter male fitness by modifying female physiology and altering egg-laying and re-mating behaviour (Gilchrist and Partridge, 2000; Friberg, 2006 but see Sirot *et al.,* 2011; Hopkins *et al.*, 2019). The relative cost of producing seminal fluid versus sperm remains unknown, but some studies suggest that seminal fluid synthesis may be costlier than sperm synthesis, and that seminal fluid can be depleted faster (e.g., Rogers *et al.,* 2005; Linklater *et al.*, 2007; Reinhardt *et al.,* 2011). This suggests that increased mating duration may be costly, and that males that mated fewer times

but for longer may have been investing more in seminal fluid transfer compared to sperm transfer. Additionally, males from the low diet also mated for significantly longer, suggesting that they may also be pursuing a different reproductive strategy involving greater investment in seminal fluid transfer over sperm transfer. However, because mating duration did not change with successive matings, this is indicative of a consistent strategy within males across all matings.

While we present clear differences in male reproductive performance that are not due to dietary and genetic effects, the underlying mechanisms remain unresolved. One factor contributing to this variation in male reproductive performance could be stochastic variation in development. Stochastic variation resulting from random differences in gene expression is a prevalent phenomenon with potentially considerable effects on phenotypes within a genotype and seemingly homogenous environments (Raj & van Oudenaarden, 2008; Feinberg & Irizarry, 2010). For example, a study by Feinberg and Izizarry (2010) found that in humans and isogenic mice, loci associated with development showed high levels of intrinsic variation in DNA methylation. Additionally, single-cell studies have shown changes in metabolism by stochastic variation in gene expression (Ozbudak *et al.*, 2002; Acar, Becskei, & van Oudenaarden, 2005). Stochastic variation in genes controlling developmental and metabolic activity could have direct consequences for male reproductive investments. Alternatively, such genes could cause subtle variation in traits like egg or pupal hatching time, with sizable down-stream effects on social interactions such as dominance and monopolisation of food that ultimately affect male condition and resources allocated to reproduction. Furthermore, such differences in male reproductive investment could also be induced by subtle environmental heterogeneity such as small-scale variation in protein concentration or social interactions. Because we did not detect any effect of male body size on mating rate, such effects were apparently not mediated by this factor (although it is possible that measuring the males that mated once or twice during the mating assay might account for substantially more variation in mating rate). Further investigations into the degree of phenotypic and behavioural variation within diets and genetic backgrounds,

and how these differences correspond to differential male reproductive investment, would shed light on the role of stochastic variation in reproduction and fitness. Uncovering sources of variation in male reproductive investment beyond dietary and genetic effects will further highlight patterns of male reproductive plasticity and shed light on how effects other than these classic predictors of condition-dependent trait expression can determine the ability of males to invest in reproduction.

Conclusions

Overall, we show that a low-nutrient larval diet results in a reduction in sperm transfer, but that males from all diets and isolines experience a similar rate of sperm depletion when mating multiply. However, across all diets and isolines, males vary substantially in mating rate, and a high mating rate was associated with faster and shorter matings, along with the transfer of more sperm. The factors resulting in such stark differences between males remain a mystery but might involve stochastic variation in developmental or metabolic conditions that affect male resource availability and allocation to reproduction, or subtle differences in the social environment of individual flies. Such results are intriguing given the paramount importance of nutrient availability and genotype as two factors influencing reproductive investment. Our results highlight the need to consider how other, less easily detectable factors, can affect reproduction and ultimately fitness.

References

ACAR, M., BECSKEI, A. & VAN OUDENAARDEN, A. (2005) Enhancement of cellular memory by reducing stochastic transitions. *Nature* **435**, 220–223.

ANDERSSON, M. (1982) Sexual selection, natural selection, and quality advertisement. *Biological Journal of the Linnean Society* **17**, 375–393.

ANDERSSON, M. (1994) *Sexual Selection*. Princeton University Press, Princeton, USA.

BATES, D., MAECHLER, M., BOLKER, B. & WALKER, S. (2015) lme4: linear mixed-effects models using Eigen and S4. *Journal of Statistical Software* **67**, 1–48.

BJORK, A., STARMER, W.T., HIGGINSON, D.M., RHODES, C.J. & PITNICK, S. (2007) Complex interactions with females and rival males limit the evolution of sperm offence and defence. *Proceedings of the Royal Society B: Biological Sciences* **274**, 1779–1788.

COSTA, A.M., ANJOS-DUARTE, C.S., RORIZ, A.K.P., DIAS, V.S. & JOACHIM-BRAVO, I.S. (2012) Male diet and age influence to inhibit female remating in *Ceratitis capitata* (Diptera: Tephritidae). *Journal of Applied Entomology* **136**, 456–463.

DANIELSSON, I. (2001) Antagonistic pre- and post-copulatory sexual selection on male body size in a water strider (*Gerris lacustris*). *Proceedings of the Royal Society B: Biological Sciences* **268**, 77–81.

DEWSBURY, D.A. (1982) Ejaculate cost and male choice. *American Naturalist* **119**, 601–610.

ENGQVIST, L. (2008) Genetic variance and genotype reaction norms in response to larval food manipulation for a trait important in scorpionfly sperm competition. *Functional Ecology* **22**, 127–133.

EVANS, J.P., RAHMAN, M.M. & GASPARINI, C. (2015) Genotype-by-environment interactions underlie the expression of pre- and post-copulatory sexually selected traits in guppies. *Journal of Evolutionary Biology* **28**, 959–972.

FALCONER, D.S. & MACKAY, T.F.C. (1996) Introduction to Quantitative Genetics.*Trends in Genetics* **12**, 202–203.

FEINBERG, A.P. & IRIZARRY, R.A. (2010) Stochastic epigenetic variation as a driving force of development, evolutionary adaptation, and disease.

Proceedings of the National Academy of Sciences **107**, 1757–1764.

FOX, J. & WEISBERG, S. (2019) An (r) companion to applied regression. Thousand Oaks, USA.

FRICKE, C., ADLER, M.I., BROOKS, R.C. & BONDURIANSKY, R. (2015) The complexity of male reproductive success: effects of nutrition, morphology, and experience. *Behavioral Ecology* **26**, 617–624.

GAGE, M.J.G. & MORROW, E.H. (2003) Experimental evidence for the evolution of numerous, tiny sperm via sperm competition. *Current Biology* **13**, 754–757.

GIENAPP, P. & MERILÄ, J. (2010) Genetic and environmental effects on a condition-dependent trait: feather growth in Siberian jays. *Journal of Evolutionary Biology* **23**, 715–723.

GRAFEN, A. (1990) Biological signals as handicaps. *Journal of Theoretical Biology* **144**, 517–546.

HILL, G.E. (2011) Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecology Letters* **14**, 625–634.

HOPKINS, B.R., SEPIL, I., THÉZÉNAS, M.L., CRAIG, J.F., MILLER, T., CHARLES, P.D., FISCHER, R., KESSLER, B.M., BRETMAN, A., PIZZARI, T. & WIGBY, S. (2019) Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* **36**, 17925–17933.

HOULE, D. (1992) Comparing evolvability and variability of quantitative traits. *Genetics* **130**, 195–204.

HUNT, J., BUSSIÈRE, L.F., JENNIONS, M.D. & BROOKS, R. (2004) What is genetic quality? *Trends in Ecology and Evolution* **19**, 329–333.

INGLEBY, F.C., HUNT, J. & HOSKEN, D.J. (2010) The role of genotype-by-environment interactions in sexual selection. *Journal of Evolutionary Biology* **23**, 2031–2045.

IWASA, Y., POMIANKOWSKI, A. & NEE, S. (1991) The evolution of costly mate preferences II. The 'handicap' principle. *Evolution* **45**, 1431–1442.

KAHRL, A.F. & COX, R.M. (2015) Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behavioral Ecology* **26**, 1502–1511.

KALDUN, B. & OTTI, O. (2016) Condition-dependent ejaculate production

affects male mating behavior in the common bedbug *Cimex lectularius*. *Ecology and Evolution* **6**, 2548–2558.

KUZNETSOVA, A., BROCKHOFF, P. & RUNE, H. (2017) lmerTest package: tests in linear mixed effects models. *Journal of Statistical Software* **82**, 1–26.

LENTH, R., SINGMANN, H., LOVE, J., BUERKNER, P. & HERVE, M. (2019) Estimated marginal means, aka least-squares means. *CRAN*, 1–74.

LINKLATER, J.R., WERTHEIM, B., WIGBY, S. & CHAPMAN, T. (2007) Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. *Evolution* **61**, 2027–2034.

LÜPOLD, S., MANIER, M.K., ALA-HONKOLA, O., BELOTE, J.M. & PITNICK, S. (2011) Male *Drosophila melanogaster* adjust ejaculate size based on female mating status, fecundity, and age. *Behavioral Ecology* **22**, 184–191.

LÜPOLD, S., MANIER, M.K., BERBEN, K.S., SMITH, K.J., DALEY, B.D., BUCKLEY, S.H., BELOTE, J.M. & PITNICK, S. (2012) How multivariate ejaculate traits determine competitive fertilization success in *Drosophila melanogaster*. *Current Biology* **22**, 1667–1672.

LÜPOLD, S., MANIER, M.K., PUNIAMOORTHY, N., SCHOFF, C., STARMER, W.T., LÜPOLD, S.H.B., BELOTE, J.M. & PITNICK, S. (2016) How sexual selection can drive the evolution of costly sperm ornamentation. *Nature* **533**, 535–538.

LÜPOLD, S., PITNICK, S., BERBEN, K.S., BLENGINI, C.S., BELOTE, J.M. & MANIER, M.K. (2013) Female mediation of competitive fertilization success in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* **110**, 10693–10698.

MACARTNEY, E.L., CREAN, A.J., NAKAGAWA, S. & BONDURIANSKY, R. (2019) Effects of nutrient limitation on sperm and seminal fluid: a systematic review and meta-analysis. *Biological Reviews* **94**, 1722–1739.

MACARTNEY, E.L., NICOVICH, P.R., BONDURIANSKY, R. & CREAN, A.J. (2018) Developmental diet irreversibly shapes male post-copulatory traits in the neriid fly *Telostylinus angusticollis*. *Journal of Evolutionary Biology* **31**, 1894–1902.

MANIER, M.K., BELOTE, J.M., BERBEN, K.S., NOVIKOV, D., STUART, W.T. & PITNICK, S. (2010) Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. *Science* **328**, 354–357.

MCGRAW, L.A., FIUMERA, A.C., RAMAKRISHNAN, M., MADHAVARAPU, S., CLARK, A.G. & WOLFNER, M.F. (2007) Larval rearing environment affects several post-copulatory traits in *Drosophila melanogaster*. *Biology Letters* **3**, 607–610.

MELO, M.C., ALMEIDA, F.R.C.L., CALDEIRA-BRANT, A.L., PARREIRA, G.G. & CHIARINI-GARCIA, H. (2014) Spermatogenesis recovery in protein-restricted rats subjected to a normal protein diet after weaning. *Reproduction, Fertility and Development* **26**, 787–796.

MULLER, K., ARENAS, L., THIÉRY, D. & MOREAU, J. (2016) Direct benefits from choosing a virgin male in the European grapevine moth, *Lobesia botrana*. *Animal Behaviour* **114**, 165–172.

NUR, N. & HASSON, O. (1984) Phenotypic plasticity and the handicap principle. *Journal of Theoretical Biology* **110**, 275–297.

O'DEA, R.E., JENNIONS, M.D. & HEAD, M.L. (2014) Male body size and condition affects sperm number and production rates in mosquitofish, *Gambusia holbrooki*. *Journal of Evolutionary Biology* **27**, 2739–2744.

OZBUDAK, E.M., THATTAI, M., KURTSER, I., GROSSMAN, A.D. & VAN OUDENAARDEN, A. (2002) Regulation of noise in the expression of a single gene. *Nature Genetics* **31**, 69–73.

PARKER, G.A. (1970) Sperm competition and its evolutionary consequences in the insects. *Biological Reviews* **45**, 535–567.

PARKER, G.A. (1990) Sperm competition games: raffles and roles. *Proceedings of the Royal Society B: Biological Sciences* **242**, 120–126.

PERRY, J.C. & ROWE, L. (2010) Condition-dependent ejaculate size and composition in a ladybird beetle. *Proceedings of the Royal Society B-Biological Sciences* **277**, 3639–3647.

POMIANKOWSKI, A. & MOLLER, A.P. (1995) A resolution of the lek paradox. *Proceedings of the Royal Society B: Biological Sciences* **260**, 21–29.

PRESTON, B.T., STEVENSON, I.R., PEMBERTON, J.M. & WILSON, K. (2001) Dominant rams lose out by sperm depletion. *Nature* **409**, 681–682.

RAHMAN, M.M., KELLEY, J.L. & EVANS, J.P. (2013) Condition-dependent expression of pre- and postcopulatory sexual traits in guppies. *Ecology and Evolution* **3**, 2197–2213.

RAJ, A. & VAN OUDENAARDEN, A. (2008) Nature, nurture, or chance:

stochastic gene expression and its consequences. *Cell* **135**, 216–226.

ROWE, L. & ARNQVIST, G. (1996) Analysis of the causal components of assortative mating in water striders. *Behavioral Ecology and Sociobiology* **38**, 279–286.

ROWE, L. & HOULE, D. (1996) The lek paradox and the capture of genetic variance by condition dependent traits. *Proceedings of the Royal Society B: Biological Sciences* **263**, 1415–1421.

SCHAALJE, G.B., MCBRIDE, J.B. & FELLINGHAM, G.W. (2002) Adequacy of approximations to distributions of test statistics in complex mixed linear models. *Journal of Agricultural, Biological, and Environmental Statistics* **7**, 512–524.

SIMMONS, L.W. & KOTIAHO, J.S. (2002) Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution* **56**, 1622–1631.

TORRES-VILA, L.M. & JENNIONS, M.D. (2005) Male mating history and female fecundity in the Lepidoptera: do male virgins make better partners? *Behavioral Ecology and Sociobiology* **57**, 318–326.

VERMEULEN, A., ENGELS, S. & SAUER, K.P. (2008) Maintenance of variance in sperm transfer rates in a scorpionfly: food availability, genetic basis, and heritability. *Behavioral Ecology and Sociobiology* **63**, 77–83.

WARD, P. (2000) Sperm length is heritable and sex-linked in the yellow dung fly (*Scathophaga stercoraria*). *Journal of Zoology* **251**, 349–353.

WEDELL, N., GAGE, M.J.G. & PARKER, G.A. (2002) Sperm competition, male prudence and sperm-limited females. *Trends in Ecology & Evolution* **17**, 313–320.

Chapter Seven

GENERAL CONCLUSION

Erin L Macartney

Since Geoff Parker's seminal papers on sperm competition and selection for investment in sperm traits (e.g., Parker, 1970, 1990a), research aiming to understand variation in sperm and semen (post-copulatory) trait expression has increased extensively. However, this relatively new research area presents many interesting questions yet to be addressed. In particular, while it is accepted that male ejaculate production is costly (e.g., Dewsbury, 1982) and should exhibit condition-dependent expression (e.g., Droney, 1998; Kahrl & Cox, 2015; Wigby *et al.*, 2016), the published literature of post-copulatory condition-dependence is strikingly complex due to the wide variety of patterns and results. This makes broad patterns of condition-dependence difficult to discern. This complexity is, in part, due to the highly multi-faceted, and often cryptic, nature of sperm and semen traits; meaning that traits might vary in the degree of condition-dependence, responses to different environments, and be subject to trade-offs. There are also substantial differences in experimental design between published studies of post-copulatory condition-dependence which are likely contributing to the difficulty in disentangling patterns of post-copulatory investment strategies. Therefore, in this thesis, I aimed to elucidate patterns of post-copulatory investment by applying life-history theory to post-copulatory traits in order to provide a clearer understanding of how variation in male condition mediates male reproductive strategies. Through this work, I show that post-copulatory traits generally adhere to predictions from life-history theory, including the allocation of limited resources to costly traits, and trade-offs among such traits. However, such patterns of post-copulatory investment depend on biologically relevant variables, including diet, genetics, access to mates, and ontogenetic constraints.

Chapter Two aimed to provide a comprehensive review of the post-copulatory condition-dependence literature by examining underlying sources of variation in sperm and semen trait responses to nutrient limitation. In this chapter, I used a meta-analysis to show that there is indeed substantial variation in the published post-copulatory trait responses to nutrient limitation. I then used meta-regression to show that much of this variation is attributed to differences between the plasticity of different sperm and semen traits, the effects of different dietary nutrients, and the ontogenetic life-stage when nutrients are

limited. I found that seminal fluid quantity and sperm quantity show consistent condition-dependent trait expression when nutrients are limited, but aspects of sperm quality, such as sperm length and viability, show weaker and less consistent condition-dependent responses. Such results suggest that investment in sperm and semen synthesis is costlier than investment in good quality sperm, potentially due to stronger selection for ejaculate quantity. Alternatively, there may be some degree of canalisation against a strong reduction in sperm quality as substantially lower quality sperm may be incapable of fertilisation and render males infertile. Additionally, I found that male post-copulatory traits respond to a wide range of nutrients, but total caloric intake and protein have the strongest and most consistent effects on post-copulatory trait expression. I also showed that the ontogenetic life-stage of nutrient limitation can affect the strength of the condition-dependent responses, but differences between the susceptibility of ontogenetic life-stages to nutrient limitation were taxon-specific. While overall, across all taxonomic groups, nutrient availability during development and during the adult stage was found to be important for post-copulatory trait expression, a closer look at effects within arthropods, mammals and fish suggested that the type of development (i.e., determinant or indeterminant growth) could be important in determining the effect of nutrient availability at different life-stages on post-copulatory investment. For example, developmental nutrient availability results in a stronger condition-dependent response in arthropods and mammals, whereas adult nutrient availability is more important for post-copulatory condition-dependence in fish. Such differences could reflect constraints in the ontogeny of post-copulatory traits between taxonomic groups. In particular, many studies of nutrient limitation on post-copulatory traits within arthropods were on holometabolous insects which have highly discrete life-stages dedicated to growth and reproduction. In contrast, fish continue to grow post reproductive maturity and thus likely allocate resources to growth and reproduction differently. Further investigation into how ontogenetic constraints affect the susceptibility of post-copulatory traits to differences in male condition will further our understanding of when post-copulatory reproductive strategies are determined.

Following on from the results found in Chapter Two, in Chapter Three, I conducted an empirical study to investigate how ontogenetic timing influences the effects of nutrient limitation on male post-copulatory traits using a native Australian neriid fly species, *Telostylinus angusticollis*. In this chapter, I examined how nutrient availability during development (i.e., as larvae) and as adults affected post-copulatory trait expression, and if there was an interaction between nutrient availability during development and as adults. I predicted that nutrient limitation at both life-stages would result in an even stronger reduction in post-copulatory trait expression compared to if nutrients were only reduced at one life-stage, and that a nutrient rich adult diet may 'rescue' effects of nutrient deprivation during development. I found that nutrient limitation during development reduced adult male testes and accessory gland size as well as sperm movement within the female reproductive tract after mating. Surprisingly, I did not detect an effect of adult diet, nor an interaction of adult diet and developmental diet. Such results demonstrate that in *T. angusticollis*, a nutrient rich (i.e., high protein) adult diet does not compensate for limited developmental nutrients nor does a reduction in nutrients at both life stages result in an even stronger reduction in post-copulatory traits compared to a situation where nutrients are only reduced at one life-stage. The strong effect of developmental nutrient availability, particularly on testes and accessory gland size, is likely, in part, due to resource allocation during metamorphosis in holometabolous insects. Holometabolous insects have highly discrete life-stages where 'decisions' of how nutrients acquired during development are allocated to adult structures are often determined during cellular reorganisation in metamorphosis (Boggs, 1981), likely explaining the strong effect on larval diet on post-copulatory traits. However, in holometabolous insects, including other Dipterans, adult nutritional resources can also be important for post-copulatory traits (e.g., Droney, 1998), and adult life-history traits can depend on a combination of developmental and adult nutritional resources (Boggs, 1981). Therefore, the ontogenetic timing of post-copulatory trait investment may vary between closely related taxa such as between different Dipteran species and such differences may be reflective of differing development times of mature sperm and accessory gland products. Furthermore, the strong effect of developmental diet on sperm movement is

unlikely to be due to a direct allocation of developmental nutritional resources during metamorphosis. Rather, such an effect may be due to some type of epigenetic programming of the cells that later develop into the male germline. The clear condition-dependent response of male post-copulatory reproductive structures and sperm movement to developmental nutrient availability demonstrates that a nutrient-rich larval diet can provide a 'silver spoon' for adult post-copulatory traits, and such effects can be irreversible. Silver spoon effects can have substantial consequences for adult fitness (Lindström, 1999; Burns *et al.*, 2012), and a reduction in testes and accessory gland size, as well as sperm movement is likely to reduce male fitness, particularly under sperm competition (e.g., Boschetto, Gasparini, & Pilastro, 2011; Bartlett *et al.*, 2017; Vellnow *et al.*, 2018). Thus, this study presents evidence that silver spoon effects can override the effects of a fully-fed diet in adulthood on male post-copulatory trait investment.

Taken together, Chapters Two and Three demonstrate the importance of environmental and physiological variation in determining how nutrient limitation affects male post-copulatory trait expression (i.e., the strength of condition-dependent responses), and raises important questions about the environments where these physiological responses evolved. Determining how differences in post-copulatory trait expression due to male condition affect male fitness, including how different ejaculate traits trade-off with each other and with pre-copulatory traits, is highly important for understanding the evolution of mating systems. For example, multiple sperm and semen traits can interact to affect male fitness (Lüpold *et al.*, 2012; Lymbery, Kennington, & Evans, 2018), and low- and high-condition individuals may invest differently in the amount of ejaculate transferred based on pre-copulatory signals and the likelihood of attaining further matings (e.g., Rowe and Arnqvist, 1996; Fricke et al., 2015). In some species, it is thought that nutrient-limited males might adopt 'sneaker' or 'satellite' reproductive strategies where such males will try to sneak copulations, and that these males may be selected to invest more in post-copulatory traits that will enhance their competitiveness in sperm competition (Parker, 1990b). Interestingly, the studies presented here suggest that nutrient-limited males may be less competitive in sperm competition, and potentially

less competitive in pre-copulatory sexual selection. Body size, often used as a signal of male condition to females, as well as sperm and semen traits were found to be strongly reduced on a nutrient limited diet in Chapter Two and Chapter Three, suggesting that such males will perform worse in both pre- and post-copulatory competition. Understanding selection for particular reproductive strategies, as well as physiological constraints on reproductive plasticity will allow us to further interpret the patterns of post-copulatory condition-dependence presented here. Furthermore, Chapter Two pointed to clear gaps in the literature on post-copulatory condition-dependence, including the lack of studies on non-model organisms. It is important to investigate reproductive strategies across a broad range of mating systems and ecologies in order to gain a more complete understanding of the evolution of reproductive strategies.

Chapters Two and Three clearly demonstrated that many post-copulatory traits are condition-dependent (albeit with substantial variation), but there has been little attempt to incorporate male investment in offspring quality through 'cryptic' non-genetic factors in the sperm and/or semen into the framework of condition-dependence and life-history theory. Consideration of the costs of male investment in offspring quality through non-genetic paternal effects has been largely limited to species in which males transfer large and nutrient-rich nuptial gifts, or invest in paternal care (e.g., Clutton-Brock, 1991; Gwynne & Simmons, 1990; Badyaev & Hill, 2002). However, over the last decade, substantial evidence has emerged that males can influence the quality of their offspring through epigenetic factors in the sperm or semen, such as non-coding RNAs, DNA methylation, and chromatin structure (Grandjean *et al.*, 2015; Milekic *et al.*, 2015; Skinner, 2016), as well as small amounts of non-nutritional proteins and peptides within the ejaculate (Chapman, 2001; Perry, Sirot, & Wigby, 2013). Yet, the costs of investing in such factors are unknown. In Chapters Four and Five, I aimed to incorporate male investment in offspring quality through 'cryptic' non-genetic factors into a condition-dependence life-history framework by asking whether investment in such non-genetic factors is costly. In Chapter Four, I argued that investment in offspring quality through investing in gametic epigenetic quality is likely to be costly and

condition-dependent, like other forms of paternal investment. I suggested that selection may favour investment in offspring quality through a high-quality sperm epigenome, particularly when such factors are tied directly to fertilisation or the risk of cuckoldry is low. I drew on the 'epigenetic clock' literature and the established effects of stress on epigenetic markers whereby age and stress appear to cause epigenetic dysregulation, and suggests that maintaining a good epigenome may impose energetic costs. I then suggested that if selection favours investment in offspring quality through investing in the gametic epigenome, such investment may evolve strongly condition-dependent expression. Additionally, I proposed experiments whereby costs of investing in offspring quality through cryptic non-genetic factors could be detected. A key idea was to test for a trade-off between investment in offspring quality and other life-history traits, and determine whether this trade-off was stronger in low-condition males.

In Chapter Five, I tested the prediction that investment in offspring quality would trade-off with other costly life-history traits by testing for a condition-dependent reduction in offspring quality with frequent mating. Few studies have addressed such a question (but see Michaud *et al.*, 2013; Mirhosseini *et al.*, 2014 for studies on coccinellid beetles), particularly in species that do not transfer nutrient rich spermatophores (but see Polak *et al.*, 2017 for a recent study on *Drosophila melanogaster*). I did this by manipulating larval diet and altering male access to mates in the neriid fly *Telostylinus angusticollis*, in which non-genetic paternal effects due to larval diet have been demonstrated (Bonduriansky & Head, 2007; Crean, Kopps, & Bonduriansky, 2014). Surprisingly, I did not detect any effects of frequent mating, nor an effect of male larval diet, on male fecundity or offspring quality. This suggests that males of high and low condition did not suffer ejaculate depletion, or males may still have had enough resources to increase ejaculate investment when provided with a single novel female (i.e., a 'Coolidge effect' (Dewsbury, 1981; Pizzari *et al.*, 2003)). However, I did detect a significantly longer mating latency in previously mated males when provided with a novel female, suggesting that previously mated males were less eager to mate after a bout of frequent mating. I then conducted a follow-up experiment to assess if previously mated males

reduced their mating rate when encountering multiple novel females and if these males were able to maintain fecundity (i.e., due to ejaculate transfer) across multiple females. If the females that mated with the males that had previously mated several times showed reduced fecundity, this would demonstrate that previously mated males do incur a cost of frequent mating and ejaculate transfer when encountering multiple new females. I found that previously mated males did not suffer a reduction in fecundity across the novel females, but males were reducing their mating rate. These results suggest that male *T. angusticollis* likely experience a trade-off between mating rate and ejaculate allocation, but that males may prioritise the quantity of ejaculate transferred over number of matings. Such patterns of ejaculate allocation could be due to the high risk of sperm competition in this species. Males are likely to be less competitive in sperm competition if they do not transfer a full ejaculate; thus, the best strategy could be to reduce mating rate in order to be competitive in sperm competition. However, because males appear to maintain full ejaculate size and composition by adjusting their mating rate, I was unable to determine if ejaculate depletion results in a corresponding decrease in offspring quality.

In both Chapters Four and Five, I attempted to incorporate investment in offspring quality through more 'cryptic' non-genetic factors into a life-history framework. Both chapters pose important questions regarding selection for investment in offspring quality and ejaculate transfer. Future studies should aim to understand environments in which selection favours investment in offspring quality versus competitiveness in sperm competition or other costly reproductive traits such as mating rate, sexual displays, or male-male combat. Also, taking a more molecular approach by using tools such as CRISPR-Cas and RNAi may prove useful in disentangling costs of investing in the production of non-genetic factors used to influence offspring quality by experimentally up- or down-regulating the expression of specific non-genetic factors. If costs of investment in non-genetic factors such as a good quality epigenome are detected, this would suggest that there could be important, condition-dependent traits that are very difficult to measure (i.e., that a key condition-dependent trait might be the pattern of DNA methylation or the

relative amounts of micro-RNAs present in a male's germ-line). Thus, extending our current understanding of what entails a condition-dependent life-history trait. While Chapter Five did not allow me to determine differential costs of different aspects of post-copulatory performance, including offspring quality, this chapter clearly demonstrates a reduction in male investment in mating rate, and suggests that this reduction occurred via a trade-off with ejaculate allocation. Such a trade-off between mating rate and ejaculate transfer is consistent with other studies on other species (reviewed in Wedell, Gage, & Parker, 2002). Future studies should further investigate how the risk of sperm competition drives trade-offs between pre- and post-copulatory investment, including investment in offspring quality. For example, populations evolving with a low risk of sperm competition may favour a higher mating rate over maintaining ejaculate stores, as ejaculate depletion may be less likely to have a drastic effect on male fitness. Differences in the risk of sperm competition may also mediate investment in the ejaculate components that determine offspring quality, thus altering the likelihood of detecting a reduction in offspring quality with frequent mating. Further research into incorporating male investment in offspring quality into life-history theory, and investigating how selection mediates investment strategies in offspring quality, traits important for sperm competition, and mating rate will provide interesting new insights into male reproductive strategies.

Finally, Chapter Six revealed the potential for stochastic variation in development or small-scale environmental variation to affect male reproductive investment. In this chapter, I tested for effects of larval diet (used to manipulate male condition) and isoline (used to test for effects of genotype) on sperm depletion in *Drosophila melanogaster*. I found that larval nutrient availability and genotype affected sperm transfer in *D. melanogaster*, with males from the low-nutrient diet transferring fewer sperm compared to males from intermediate and high-nutrient diets. Males also experienced sperm depletion and an increase in mating latency with successive matings, demonstrating that males were incurring a cost of mating on sperm transfer and ability to rapidly initiate mating. However, the rate of sperm depletion and increase in mating latency was not affected by diet or genotype, meaning that sperm depletion

was not condition-dependent. Interestingly, I detected clear differences in male mating rate that were not due to diet or genotype, and found that mating rate was highly correlated to other aspects of male mating behaviour and sperm transfer. Males that mated more also had shorter mating latencies and shorter mating durations, and transferred more sperm, meaning that some males were better able to invest in both mating number and sperm transfer. Thus, some males do not appear to experience a trade-off between mating rate and sperm transfer. However, the cause of such clear differences in male reproductive behaviour and performance is not known. I suggested that such differences may be due to stochastic variation in gene expression or due to small-scale variation not accounted for by my experimental design. For example, gene expression can vary within seemingly homogenous environments and genotypes (e.g., Raj & van Oudenaarden, 2008; Feinberg & Irizarry, 2010), raising important questions of how random variation in gene expression can contribute to variation in life-history traits important for fitness. Furthermore, understanding how subtle environmental heterogeneity, such as small-scale differences in protein availability or social interactions contribute to differences in life-history traits is also important for understanding the clear differences in male reproductive performance presented here. Future studies should investigate the degree of phenotypic variation in physiological, morphological, and behavioural traits within homogenous environments and genotypes, and assess how such variation can contribute to differences in reproductive strategies. Further studies into the causes of this variation is also needed by determining of such patterns are truly due to stochastic variation, or determined by micro-environmental variations (i.e., nutrient concentrations or social interactions). Such a study would provide interesting new insights into how subtle and apparently random variation can have down-stream effects on life-history strategies and fitness.

Overall, this thesis highlights many interesting patterns of male post-copulatory investment and condition-dependence that, I hope, will increase our understanding of how environmental, physiological, and genetic variation can alter post-copulatory trait expression. Determining which selection environments result in particular reproductive strategies and taking a

multivariate approach to understand how the condition-dependence of multiple pre- and post-copulatory traits interact to affect male fitness will help us to understand the processes that lead to the patterns of reproductive investment reported throughout this thesis. There are also post-copulatory traits that are largely unexplored in ecological and evolutionary contexts; in particular, non-genetic paternal effects conferred through 'cryptic' factors in the ejaculate remain poorly understood and have rarely been investigated as part of the male reproductive strategy. Additionally, finding methods to overcome logistical issues such as determining paternity/differentiating sperm from different males is important for determining how post-copulatory condition-dependence affects male fitness under competitive settings. This was an issue experienced in the empirical studies on *Telostylinus angusticollis*, but it is likely possible to complete such studies on the genetically engineered *Drosophila melanogaster* used in Chapter Six and I hope to complete such a study in the near future. Developing techniques that would make this type of study possible in non-model systems such as *T. angusticollis*, would be highly valuable for furthering our understanding of how males of high- and low-condition perform under sperm-competition across a range of taxa and mating systems. Overall, exploring selection on post-copulatory investment strategies and additional sources of variation on post-copulatory traits, including non-genetic paternal effects, is key to furthering our understanding of ejaculate evolution, reproductive investment strategies, and differences in male fitness. I hope that this thesis will prove valuable in furthering our understanding of male post-copulatory condition-dependence.

References

BADYAEV, A. V & HILL, G.E. (2002) Paternal care as a conditional strategy: distinct reproductive tactics associated with elaboration of plumage ornamentation in the house finch. *Behavioral Ecology* **13**, 591–597.

BARTLETT, M.J., STEEVES, T.E., GEMMELL, N.J. & ROSENGRAVE, P.C. (2017) Sperm competition risk drives rapid ejaculate adjustments mediated by seminal fluid. *eLife* **6**, 1–23.

BOGGS, C.L. (1981) Nutritional and life-history determinants of resource-allocation in holometabolous insects. *American Naturalist* **117**, 692–709.

BONDURIANSKY, R. & HEAD, M. (2007) Maternal and paternal condition effects on offspring phenotype in *Telostylinus angusticollis* (Diptera: Neriidae). *Journal of Evolutionary Biology* **20**, 2379–2388.

BOSCHETTO, C., GASPARINI, C. & PILASTRO, A. (2011) Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology* **65**, 813–821.

BURNS, J.G., SVETEC, N., ROWE, L., MERY, F., DOLAN, M.J., BOYCE, W.T. & SOKOLOWSKI, M.B. (2012) Gene-environment interplay in *Drosophila melanogaster*: chronic food deprivation in early life affects adult exploratory and fitness traits. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 17239–17244.

CHAPMAN, T. (2001) Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity* **87**, 511–521.

CREAN, A.J., KOPPS, A.M. & BONDURIANSKY, R. (2014) Revisiting telegony: offspring inherit an acquired characteristic of their mother's previous mate. *Ecology Letters* **17**, 1545–1552.

DEWSBURY, D.A. (1982) Ejaculate cost and male choice. *American Naturalist* **119**, 601–610.

DEWSBURY, D.A.A. (1981) Effects of novelty on copulatory behavior: the coolidge effect and related phenomena. *Psychological Bulletin* **89**, 464–482.

DRONEY, D.C. (1998) The influence of the nutritional content of the adult male diet on testis mass, body condition and courtship vigour in a Hawaiian *Drosophila*. *Functional Ecology* **12**, 920–928.

FEINBERG, A.P. & IRIZARRY, R.A. (2010) Stochastic epigenetic variation as a driving force of development, evolutionary adaptation, and disease.

Proceedings of the National Academy of Sciences **107**, 1757–1764.

FRICKE, C., ADLER, M.I., BROOKS, R.C. & BONDURIANSKY, R. (2015) The complexity of male reproductive success: effects of nutrition, morphology, and experience. *Behavioral Ecology* **26**, 617–624.

GRANDJEAN, V., FOURRE, S., DE ABREU, D.A.F., DERIEPPE, M.A., REMY, J.J. & RASSOULZADEGAN, M. (2015) RNA-mediated paternal heredity of diet-induced obesity and metabolic disorders. *Scientific Reports* **5**, 1–9.

KAHRL, A.F. & COX, R.M. (2015) Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behavioral Ecology* **26**, 1502–1511.

LINDSTRÖM, J. (1999) Early development and fitness in birds and mammals. *Trends in Ecology and Evolution* **14**, 343–348.

LÜPOLD, S., MANIER, M.K., BERBEN, K.S., SMITH, K.J., DALEY, B.D., BUCKLEY, S.H., BELOTE, J.M. & PITNICK, S. (2012) How multivariate ejaculate traits determine competitive fertilization success in *Drosophila melanogaster*. *Current Biology* **22**, 1667–1672.

LYMBERY, R.A., KENNINGTON, W.J. & EVANS, J.P. (2018) Multivariate sexual selection on ejaculate traits under sperm competition. *The American Naturalist* **192**, 94–104.

MILEKIC, M.H., XIN, Y., DONNELL, A.O., KUMAR, K.K., MALASPINA, D., MOORE, H., BRUNNER, D., GE, Y., EDWARDS, J. & PAUL, S. (2015) Age-related sperm DNA methylation changes are transmitted to offspring and associated with abnormal behavior and dysregulated gene expression. *Molecular Psychiatry* **20**, 995–1001.

PARKER, G.A. (1970) Sperm competition and its evolutionary consequences in the insects. *Biological Reviews* **45**, 535–567.

PARKER, G.A. (1990a) Sperm competition games: raffles and roles. *Proceedings of the Royal Society B: Biological Sciences* **242**, 120–126.

PARKER, G.A. & PIZZARI, T. (2010) Sperm competition and ejaculate economics. *Biological Reviews* **85**, 897–934.

PERRY, J.C., SIROT, L. & WIGBY, S. (2013) The seminal symphony: how to compose an ejaculate. *Trends in Ecology & Evolution* **28**, 414–422.

PIZZARI, T., CORNWALLIS, C.K., HANNE, L., JAKOBSSON, S. & BIRKHEAD, T.R. (2003) Sophisticated sperm allocation in male fowl. *Nature* **426**, 70–

74.

RAJ, A. & VAN OUDENAARDEN, A. (2008) Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell* **135**, 216–226.

ROWE, L. & ARNQVIST, G. (1996) Analysis of the causal components of assortative mating in water striders. *Behavioral Ecology and Sociobiology* **38**, 279–286.

SKINNER, M.K. (2016) Epigenetic transgenerational inheritance. *Nature Reviews* **12**, 68–70.

CLUTTON-BROCK , T.H. (1991) *The Evolution of Parental Care*. Princeton University Press, Princeton, USA.

VELLNOW, N., MARIE-ORLEACH, L., ZADESENETS, K.S. & SCHÄRER, L. (2018) Bigger testes increase paternity in a simultaneous hermaphrodite, independently of the sperm competition level. *Journal of Evolutionary Biology* **31**, 180–196.

WEDELL, N., GAGE, M.J.G. & PARKER, G.A. (2002) Sperm competition, male prudence and sperm-limited females. *Trends in Ecology & Evolution* **17**, 313–320.

WIGBY, S., PERRY, J.C., KIM, Y.H. & SIROT, L.K. (2016) Developmental environment mediates male seminal protein investment in *Drosophila melanogaster*. *Functional Ecology* **30**, 410–419.