

# **Sperm competition and gamete interactions in a marine broadcast spawner**

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This thesis is presented for the degree of Doctor of Philosophy of The University of Western  
Australia

School of Biological Sciences  
Centre for Evolutionary Biology

2018

## THESIS DECLARATION

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I, Rowan Lymbery, certify that:

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The research reported in this thesis involved invertebrates (mussels), which do not require approval by The University of Western Australia Animal Ethics Committee.

The research involving invertebrates reported in this thesis followed The University of Western Australia and national standards for the care and use of laboratory animals.

The following approvals were obtained prior to commencing the relevant work described in this thesis:

- Department of Fisheries, Government of Western Australia Research Exemption #2718
- Department of Transport, Government of Western Australia Temporary Land/Water Permits (Woodman Point, Jervoise Bay and Claremont Jetty, Point Walter)

The work described in this thesis was funded by ARC Discovery grants awarded to Jonathan Evans and Jason Kennington (DP150103266 and DP170103290). This thesis was supported by the Hackett Postgraduate Research Scholarship and the Bruce and Betty Green Postgraduate Research Top-Up Scholarship, awarded to Rowan Lymbery.

Technical assistance was kindly provided by the Australian Genome Research Facility (AGRF) for RNA sequencing and bioinformatic support, as described in Chapter 4; Yvette Hitchen for mitochondrial DNA analysis, as described in Chapter 3; and Cameron Duggin for practical assistance with mussel collections in Chapters 2, 4 and 5.

This thesis contains published work and/or work prepared for publication, some of which has been co-authored.

Signature: 

Date: 14/12/2017

## ABSTRACT

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Sexual selection, which acts on variation in traits that influence reproductive success, almost certainly began in the sea with broadcast spawning organisms. The combination of relative immobility and external fertilisation in ancestral taxa means there was likely limited opportunity for the pre-mating forms of sexual selection described by Darwin (male-male competition for mates and female mate choice). Instead, mass spawning events and the co-occurrence of gametes from multiple individuals likely generated intense post-ejaculatory sexual selection, in the form of (a) competition for fertilisations among ejaculates from rival males (sperm competition), and (b) opportunities for females (or eggs) to bias fertilisations through gamete-level mate choice. Both processes (sperm competition and gamete-level mate choice) remain pervasive forms of sexual selection in most sexually reproducing taxa. Given their ancestral mode of reproduction, the mechanisms underlying gamete interactions in broadcast spawners have direct relevance to a broad range of species.

In this thesis, I applied a range of novel experimental techniques to investigate the mechanisms and outcomes of gamete-level sexual selection in the broadcast spawning mussel, *Mytilus galloprovincialis*. In my first experimental Chapter, I exploited a unique aspect of bivalve biology, the paternal inheritance of mitochondrial DNA (mtDNA), to develop a method for tracking the outcomes of sperm competition. Specifically, I used the vital mitochondrial dye MitoTracker to measure the success of sperm from individual males when they compete with those from rivals to fertilise eggs. This technique was shown to have no effect on sperm motility or sperm competitive fertilisation success.

In my second experimental Chapter, I applied the mitochondrial dye technique in sperm competition trials to test a putative mechanism of gamete-level mate choice: the differential attraction of sperm by egg-derived chemicals. This phenomenon, known as sperm chemotaxis, is taxonomically ubiquitous, but has not been examined previously in the context of sperm competition. Recent studies in *M. galloprovincialis* have demonstrated that chemoattractants from different females have differential effects on the swimming direction, motility characteristics, and physiology of sperm from different males. Here, I used the MitoTracker technique in combination with competitive chemotaxis trials to show that competitive fertilisation success is moderated by differential sperm chemotaxis. Moreover, I explored the genetic patterns

underlying chemoattractant-driven fertilisation biases, finding that fertilisation biases reflect nuclear genetic relatedness and phylogenetic mtDNA lineage. Together, these findings suggest that females derive genetic benefits from gamete-level mate choice.

In my third experimental Chapter I explored the molecular mechanisms underlying changes in sperm phenotype in response to egg chemoattractants. Specifically, RNA-sequencing techniques were used to compare sperm gene expression in the presence and absence of chemoattractants. I found 57 differentially expressed genes between control sperm (exposed to seawater alone) and treated sperm (exposed to seawater and egg chemoattractants). A number of these genes matched functional protein domains that may be involved in known responses to chemoattractants, including signal reception, sperm motility, capacitation and the acrosome reaction. Moreover, this is the first evidence in any system of differential gene expression in mature, ejaculated sperm, which could have widespread implications for our understanding of the mechanisms that control sperm function and behaviour.

In my final experimental Chapter, I explored overall patterns of selection on ejaculate traits under sperm competition. In this study I applied the mitochondrial dye technique developed previously, during experimental trials that simulate large multi-male, multi-female spawning events, thereby deriving measures of male reproductive success across a range of rival ejaculates and females. By measuring a range of sperm motility and morphology traits and applying the statistical techniques of multivariate selection, complex patterns of non-linear selection were revealed on combinations of ejaculate traits. Specifically, I found non-linear selection against divergent combinations of sperm length, velocity and swimming path linearity, which likely reflect the way different swimming strategies allow sperm to locate and track eggs. Importantly, these results demonstrate overall patterns of selection on ejaculate traits across reproductive scenarios, suggesting the potential for adaptive evolution of ejaculates under sperm competition.

Overall, this thesis addresses important unresolved questions in post-ejaculatory sexual selection research, and demonstrates a series of novel gamete interactions. Moreover, I anticipate that the experimental techniques developed throughout the thesis could be adapted for a wide range of systems, with the potential to greatly improve our understanding of gamete-level sexual selection.

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## ACKNOWLEDGEMENTS

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First and foremost, I would like to thank my supervisors, Jon Evans and Jason Kennington, for their incredible support and commitment. They always placed a high priority on my project, and their feedback was unfailingly swift and valuable. I consider myself lucky to count them as supervisors, mentors and friends, and look forward to continuing work with them both. I would also like to thank the rest of the EVOLab, past, present and honorary, for their support and friendship; Clelia, Jen, Cam, Angie, Jukka, Moshi, Tabs, Ryan and Federica to name only a few. In particular, thanks go to Clelia Gasparini and Angie Eads for their practical guidance when I was starting this project. And of course, many thanks to Cam Duggin for help with lab and fieldwork, but also for the coffees, lunches, great conversation and unparalleled collection of quotes.

Thanks also go to numerous other people who provided practical support throughout this project. To my research assistants for the multivariate selection experiment, Brighton Downing and Emma Daymond, thank you for the long days in the lab! I am also very grateful to Yvette Hitchen, Sherralee Lukehurst, Ryan Dosselli and Joanito Liberti for advice, guidance and assistance with the molecular aspects of the project. I would also like to thank the Australian Genome Research Facility staff for technical support with RNA sequencing and bioinformatics, particularly Juan Montenegro for working with me to develop the bioinformatics pipeline.

I would like to thank everyone in the Centre for Evolutionary Biology, my office mates, and my fellow Animal Biology postgrads, for their support and friendship. It has been an incredibly dynamic research environment to work in. In particular, thanks go to Bruno Buzatto and Jake Berson for interesting and valuable discussions about R and statistical analyses, Blair Bentley for advice and discussions about transcriptomics, as well as Ben Ashton and Sam Lymbery for helping stay sane during thesis writing with weekly squash games. Special thanks to Leigh Simmons and Joe Tomkins for their unfailing commitment to postgraduate support.

Thank you also to the friends and family who have supported me throughout my undergraduate and postgraduate journey. In particular, the extended undergrad Zoology crew, who have remained some of my closest friends even when I have been buried in my thesis, and the many friends I have made among my postgrad and postdoc colleagues – there are too many to name, but you know who you are and thanks for being legends! To my family, particularly Mum, Dad and Sam, thank you for the unwavering support in all my endeavours. Words cannot express how

grateful I am for everything you do, and you all provide amazing guidance and inspiration in my career pursuits. And of course, many thanks to you all for help with compiling, proof-reading and reference-checking of this thesis, and to Dad for many helpful comments on manuscripts and drafts. Last, but most certainly not least, my wife Ashley (and of course our best buddy Dexter). Thank you always for your unconditional love, encouragement and support (from South Africa to the USA to Australia). I cannot say how grateful I am to have you with me on this journey; you have made it all worthwhile.

The research in this thesis was supported by an Australian Government Research Training Program (RTP) Scholarship, with further funding provided by the Australian Research Council. Stipend support was provided by the Hackett Postgraduate Research Scholarship and the Bruce and Betty Green Postgraduate Research Top-Up Scholarship



*In memory of Jeremy*

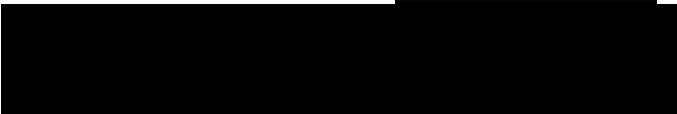
*(1991-2011)*


*"Here's to being pompous."*

## AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS

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This thesis contains a combination of work that has been published, is under consideration for publication, and has been prepared for publication. Specifically, Chapters 2 and 3 have been published in peer-reviewed journals; Chapter 4 has been prepared as a manuscript for submission to a peer-reviewed journal; Chapter 5 is under editorial consideration as an invited resubmission in a peer-reviewed journal.

<b>Details of the work:</b> Lymbery, R. A., Kennington, W.J. and Evans, J. P. 2016 Fluorescent sperm offer a method for tracking the real-time success of ejaculates when they compete to fertilise eggs. <i>Scientific Reports</i> 6: 22689.
<b>Location in thesis:</b> Chapter 2
<b>Student contribution to work:</b> Lead author, responsible for experimental design, data collection, statistical analysis and writing. Overall contribution over 85%.
<b>Co-author signatures and dates:</b>  Jonathan Evans                      Jason Kennington 14/12/17                                14/12/17

<b>Details of the work:</b> Lymbery, R. A., Kennington, W.J. and Evans, J. P. 2017 Egg chemoattractants moderate intraspecific sperm competition. <i>Evolution Letters</i> 1: 317-327.
<b>Location in thesis:</b> Chapter 3
<b>Student contribution to work:</b> Lead author, responsible for experimental design, data collection, statistical analysis and writing. Overall contribution over 85%.
<b>Co-author signatures and dates:</b>  Jonathan Evans                      Jason Kennington 14/12/17                                14/12/17

<p><b>Details of the work:</b>  Lymbery, R. A., Evans, J. P and Kennington, W.J. <i>In prep.</i> Egg chemoattractants induce differential gene expression in sperm</p>				
<p><b>Location in thesis:</b>  Chapter 4</p>				
<p><b>Student contribution to work:</b>  Lead author, responsible for experimental design, data collection, statistical analysis and writing. Overall contribution over 85%.</p>				
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<p><b>Details of the work:</b>  Lymbery, R. A., Kennington, W.J. and Evans, J. P. 2018. Multivariate sexual selection on ejaculate traits under sperm competition. <i>American Naturalist</i>. <i>In press.</i></p>				
<p><b>Location in thesis:</b>  Chapter 5</p>				
<p><b>Student contribution to work:</b>  Lead author, responsible for experimental design, data collection, statistical analysis and writing. Overall contribution over 85%.</p>				
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<p>I, Jonathan Evans certify that the student statements regarding their contribution to each of the works listed above are correct.</p> <div style="background-color: black; width: 100%; height: 40px; margin-bottom: 5px;"></div> <p>Coordinating supervisor signature:  Date: 14/12/2017</p>



## CHAPTER ONE

### General Introduction

*“With animals belonging to the lower classes, the two sexes are not rarely united in the same individual, and therefore secondary sexual characters cannot be developed. In many cases where the sexes are separate, both are permanently attached to some support, and the one cannot search or struggle for the other. Moreover it is almost certain that these animals have too imperfect senses and much too low mental powers to appreciate each other’s beauty or other attractions, or to feel rivalry.”*

Charles Darwin (1871)



### **1.1 Sexual selection: Darwin's dilemma**

The concept of sexual selection was developed by Darwin (1859, 1871) as an extension of his theory of evolution by natural selection. He was interested in explaining within-species sexual dimorphism, which often manifests as traits in one sex (typically males) that appear to be detrimental to survival. For example, Darwin noted that the ornamental tail and wing feathers of male birds such as pheasants, widow-birds and peacocks had apparently detrimental effects on the efficiency of flying and running, leaving them vulnerable to predation (Darwin 1871). Darwin's solution to this problem was sexual selection, which he described as depending on "the advantage that certain individuals have over others of the same sex and species, solely in respect of reproduction" (Darwin 1871, p. 231). He proposed that many elaborate male traits functioned as weapons or ornaments that confer a reproductive advantage during competition for mates, through male-male contests or attraction of females, respectively.

Since Darwin's revolutionary insights, we now understand that sexual selection arises because of differential investment in reproduction between the sexes (Bateman 1948; Trivers 1972; Emlen and Oring 1977; Clutton-Brock and Vincent 1991). For example, Bateman's (1948) influential experiments on *Drosophila melanogaster* were the first to demonstrate that a male's fitness increases more strongly with increased number of matings than females, which he attributed to males investing less in gametes than females (i.e. anisogamy). This explained Darwin's (1871) observation that males often compete for mates and females often exercise mate choice. Bateman's (1948) theory was extended by Trivers (1972), who recognised that differences in parental investment by males and females were also key determinants of the relative strength of sexual selection on the sexes. We now know that a range of factors can contribute to intersexual variation in reproductive investment (Jennions and Kokko 2010; Parker 2014). This can lead to deviations from the usual patterns of male-male competition and female choice, because in some cases females invest less in reproduction than males (reviewed by Clutton-Brock 2007). Concepts of sexual selection have become extremely powerful tools for explaining the occurrence of traits such as exaggerated weapons and ornaments in many species (Andersson 1994; Andersson and Simmons 2006; McCullough et al. 2016).

### **1.2 Post-ejaculatory sexual selection**

A major turning point in the field of sexual selection was the recognition that sexual selection can continue after copulation or ejaculation. This was stimulated by Parker's (1970) landmark review

of sperm competition in insects, where he argued that multiple mating by females (i.e. polyandry) would lead to sperm competition, where the ejaculates of rival males compete to fertilise a female's eggs. Since Parker's (1970) pioneering work, studies employing a range of behavioural and molecular tools have confirmed that polyandry is taxonomically widespread (Smith 1984; Birkhead and Møller 1998; Simmons 2001; Griffith et al. 2002). While most of this evidence exists for internally-fertilising species, it has also become clear that many externally-fertilising taxa engage in multi-individual synchronous spawnings, which also leads to competition for fertilisations among multiple ejaculates (Levitan 1998; Parker et al. 2017).

A decade after Parker (1970) conceptualised sperm competition as the post-ejaculatory analogue of male-male mating competition, Thornhill (1983) provided the first empirical evidence that females can manipulate sperm competition to suit their own reproductive interests, a phenomenon he coined 'cryptic female choice' because it occurs after mating and often out of sight of the human observer. Specifically, Thornhill (1983) described the manipulation of sperm uptake and oviposition during and after multiple mating by female scorpionflies, *Harbobitticus nigriceps*. However, it was not until Eberhard's (1996) review that the broader research community became aware of this phenomenon of cryptic female choice, where females exercise post-ejaculatory control over the fertilisation outcomes of sperm competition (analogous to female mate choice). Sperm competition and cryptic female choice are now recognised as important forms of sexual selection across most sexually-reproducing taxa (Simmons 2001; Birkhead and Pizzari 2002; Eberhard 2009), and are typically categorised as 'post-copulatory' sexual selection as distinct from Darwinian 'pre-copulatory' mating competition and mate choice. Here, I follow Parker (2014) in using the terms pre- and post-ejaculatory, to include externally-fertilising taxa that do not pair or copulate during reproduction.

#### *Sperm competition and selection on ejaculates*

Initially, much of the empirical research on post-ejaculatory sexual selection focused on the selective outcomes of sperm competition for males. Accordingly, early sperm competition theory generated the prediction that selection should favour male traits that prevent or minimise competition with rival ejaculates (Parker 1970). In support of this prediction, considerable empirical evidence has accumulated to show that males of numerous taxa engage in behavioural mate guarding to reduce the probability of further matings (Birkhead et al. 1985; Sherman 1989; Sakaluk 1991; Alonzo and Warner 2000; Schöfl and Taborsky 2002). For example, male firebugs



(*Pyrrhocoris apterus*) more than double the length of time spent in tandem with females when the operational sex ratio (ratio of sexually receptive males to females) is male-biased compared to when it is female-biased (Schöfl and Taborsky 2002). Males can also manipulate female receptivity to re-mating through physical or chemical mechanisms; for example, the seminal fluid sex peptide in *Drosophila melanogaster* can delay female re-mating for 1-2 days (Chapman et al. 2003; Fricke et al. 2009).

When sperm competition cannot be avoided, selection should favour male traits that allow ejaculates to outcompete those of rivals for fertilisation (Parker 1998). Arguably the best example of this is through an increased expenditure in sperm production; ejaculates comprising greater numbers of sperm typically achieve higher competitive success than those with fewer sperm (e.g. Martin et al. 1974; Gage and Morrow 2003). This can lead to selection on investment in sperm numbers (Gage 1995; Gage et al. 1995; Møller and Briskie 1995; Stockley et al. 1997), as for example in the moth *Plodia interpunctella*, where males have larger testes at higher population densities (Gage 1995) and evolve increased sperm numbers per ejaculate under male-biased sex ratios (Ingleby et al. 2010).

More recent attention has turned to the evolution of individual sperm traits such as sperm velocity, sperm morphology and sperm viability (collectively referred to as sperm quality; Snook 2005), with varying results. For example, sperm velocity can positively influence fertilisation success (reviewed in Simmons and Fitzpatrick 2012), although recent studies have found exceptions. For example, slower sperm are more competitively successful at being retained by females in *D. melanogaster* (Lüpold et al. 2012), and at achieving fertilisations in the frog *Crinia georgiana* (Dziminski et al. 2009). Similarly, sperm size may influence competitive fertilisation success via an association with velocity, i.e. sperm with longer flagella being faster swimmers (Fitzpatrick and Lüpold 2014). However, the evidence linking sperm length to velocity is inconsistent among taxa with different mating systems, and a recent multi-species study reported positive relationships between sperm head-flagellum length (H:F) ratio and sperm velocity for internal fertilisers, and negative relationships for external fertilisers (Simpson et al. 2014). Additionally, there has also been recent interest in other ejaculate components that might influence sperm survival and competitiveness. For example, seminal fluid might enhance sperm velocity (e.g. *Atta columbica*; den Boer et al. 2008; *D. melanogaster*; Holman 2009; *Teleogryllus oceanicus*; Simmons and Beveridge 2011), or detrimentally affect rival sperm performance (e.g.

*Apis mellifera*, *Acromyrmex echinator*, *Atta columbica*; den Boer et al. 2010; *Zosterisessor ophiocephalus*; Locatello et al. 2013) These diverse and often conflicting empirical findings suggest selection on ejaculates is extremely complex.

Several factors may account for difficulties in elucidating patterns of selection on ejaculate traits. First, confounding variables can make the estimation of competitive fertilisation success challenging. For example, a landmark study by Gilchrist and Partridge (1997) showed that variation in the ability of *D. melanogaster* to displace rival sperm, estimated through offspring paternity biases, was almost entirely explained by variation in offspring viability. Thus, studies that rely on paternity data to infer patterns of competitive fertilisation success risk confounding post-zygotic factors (e.g. differential embryo survival) with those driving fertilisation biases (Olsson et al. 1999; Evans et al. 2007; García-González 2008a). Second, intrinsic sperm quality traits might be modified by the female reproductive environment, and competitive success might depend on complex interactions between sperm and the female environment in which they compete (Fitzpatrick and Lüpold 2014; see below, '*Consequences of ejaculate-female interactions for selection on sperm*'). Third, there is a growing recognition that ejaculates function as 'integrated phenotypes', and thus that selection on ejaculates will favour combinations of sperm traits rather than individual characteristics (Pizzari and Parker 2009). Elucidating patterns of multivariate sexual selection on ejaculates is recognised as critical prerequisite for improving our understanding of sperm competition (Pizzari and Parker 2009).

#### *Mechanisms of cryptic female choice*

The accumulating evidence that females from a wide range of taxa influence the outcomes of sperm competition (e.g. Clark et al. 1999; Nilsson et al. 2003; Pilastro et al. 2004; Lovlie et al. 2013) has seen a considerable increase in empirical research on cryptic female choice. Although evidence for cryptic female choice is now overwhelming, the mechanisms underlying cryptic female choice remain unclear in most species, most likely because these processes typically occur within the female reproductive tract (Firman et al. 2017). Nevertheless, proximate mechanisms of cryptic female choice have been uncovered in some species, and these include processes of removing sperm from the reproductive tract by female internal fertilisers. For example, female fowl (*Gallus gallus*) differentially eject sperm based on male status following multiple matings, preferentially retaining sperm from dominant males (Pizzari and Birkhead 2000; see also Dean et al. 2011). Similarly, a series of elegant experiments on *D. melanogaster* have shown that females

influence sperm competition through sperm ejection (Manier et al. 2010). When female *D. melanogaster* mate sequentially to two males, the second male's sperm displaces rival male sperm already present in the female's sperm storage organs (Manier et al. 2010), and females can mediate the relative success of first or second male sperm by varying the timing of ejection of excess and displaced sperm (Lüpold et al. 2013). Therefore, differential sperm removal from the female reproductive tract appears to be an important mechanism of cryptic female choice in taxonomically diverse internal fertilisers.

More recently, there has been growing interest in the chemical mediation of sperm swimming behaviour by female reproductive fluids, which are secretions released by the female reproductive tract, accessory glands, or eggs (Firman et al. 2017). The moderation of sperm performance by substances released by females or eggs is a taxonomically widespread phenomenon across both internal and external fertilisers (Miller 1985; Eisenbach and Giojalas 2006; Schnakenberg et al. 2011; Beekman et al. 2016; Degner and Harrington 2016). For example, the ovarian fluid (OF) of female fishes, which is retained in the reproductive tract for internal fertilisers or spawned with eggs as an extracellular matrix for external fertilisers, can differentially affect sperm swimming performance of different males (Urbach et al. 2005; Rosengrave et al. 2008; Gasparini and Pilastro 2011; Butts et al. 2012). In sister species of externally-fertilising salmonids, Yeates et al. (2013) found that OF preferentially attracts sperm from the same species, resulting in competitive fertilisation preferences toward conspecifics. Moreover, in the internally fertilising guppy (*Poecilia reticulata*), OF increases the velocity of sperm from unrelated conspecific males over those from related conspecifics (siblings), which explains competitive paternity biases toward unrelated males when artificial insemination is used to compete related and unrelated sperm (Gasparini and Pilastro 2011). Despite this progress, however, the effect of female reproductive fluids on competitive fertilisations have yet to be investigated in a wider range of taxa. The ubiquity of such fluids suggests they have the potential to be widespread mediators of sperm competition.

#### *Adaptive benefits of multiple mating and cryptic female choice*

The adaptive basis of cryptic female choice remains unclear in many species. Cryptic female choice is expected to provide reproductive benefits for females when there are constraints on pre-ejaculatory mate choice (Firman et al. 2017). Specifically, theory predicts that females could receive fitness benefits for offspring by ensuring that their eggs are fertilised by sperm from genetically better (additive genetic benefits) or more compatible males (non-additive genetic

benefits) (Zeh and Zeh 1996, 1997; Tregenza and Wedell 2000; Neff and Pitcher 2005). This could occur through multiple mating if sperm from genetically better/compatible males achieve some proportion of fertilisations (Curtisinger 1991; Keller and Reeve 1995; Yasui 1997; Simmons 2005; Slatyer et al. 2012). Cryptic female choice is likely to enhance the accumulation of such benefits by (a) providing a selectively challenging environment that ensures the competitively 'best' sperm achieve fertilisation (directional cryptic female choice), or (b) promoting fertilisations by sperm from genetically compatible males (non-directional cryptic female choice) (Sivinski 1984; Jennions and Petrie 2000; Birkhead and Pizzari 2002; Simmons 2005).

Testing hypotheses for the adaptive value of cryptic female choice has been the focus of much empirical research, and several studies suggest that benefits of genetic compatibility have the potential to be widespread. For example, a pioneering study using multiple mating trials in field crickets (*Gryllus bimaculatus*) demonstrated that females mated to non-sibling males had higher egg hatching success than those mated to sibling males, and females mated to one sibling and one non-sibling could avoid the detrimental effects of sibling fertilisations (Tregenza and Wedell 2002). Similar preferences for sperm from unrelated males during competitive fertilisations have since been found in vertebrates (e.g. *P. reticulata*; Gasparini and Pilastro 2011; *Mus domesticus*; Firman and Simmons 2015). Additionally, females might base gamete-level mate choice on beneficial combinations of specific loci. For example, several studies have found post-ejaculatory preferences for sperm from males that are genetically similar (e.g. in *Salmo salar*; Yeates et al. 2009) or dissimilar (e.g. *G. gallus*; Lovlie et al. 2013) at the major histocompatibility complex, a set of genes involved in immune responses in vertebrates. However, distinguishing among competing hypotheses for the genetic benefits of cryptic female choice has proven empirically challenging in most systems (Slatyer et al. 2012; Firman et al. 2017), and as I note above it is often difficult to separate differential sperm competitive success (measured as paternity biases) from differential offspring fitness (García-González 2008a). For example, the estimation of female-mediated paternity biases among sperm from sibling and non-sibling males can be confounded by inbreeding effects on embryo mortality (Olsson et al. 1999). Therefore, it is often difficult to unambiguously demonstrate fertilisation biases toward sperm that provide genetic benefits to females.

### *Consequences of ejaculate-female interactions for selection on sperm*

As a consequence of the widespread occurrence of cryptic female choice and female mediation of sperm performance, post-ejaculatory sexual selection on males is unlikely to be attributable solely to competitive interactions among ejaculates (Pitnick and Hosken 2010). Female effects might influence patterns of post-ejaculatory selection on ejaculates by modifying both sperm phenotypes and competitive fitness outcomes (Pitnick et al. 2009; Fitzpatrick and Lüpold 2014), as in the examples given above for the differential mediation of sperm swimming characteristics and competitive success by female salmonid and poeciliid fish (Rosengrave et al. 2008; Gasparini and Pilastro 2011; Yeates et al. 2013). In the case of non-directional female fertilisation preferences for sperm from compatible males, post-ejaculatory selection on specific male traits may be constrained (Neff and Pitcher 2005; Pitnick and Hosken 2010). Even when cryptic female choice is directional, the resulting selection on ejaculate phenotypes may differ from theoretical expectations based on sperm competition. For example, Alonzo et al. (2016) found that by altering sperm swimming velocity through ovarian fluid, females of the wrasse *Symphodus ocellatus* decrease the relative importance of sperm number in determining paternity, thus favouring large nesting males that produce fewer, faster sperm than their sneaker counterparts. Similarly in *Drosophila*, rather than simply selection for numerous small sperm under sperm competition, there is post-ejaculatory sexual selection for increased sperm size driven by the evolution of longer female reproductive tracts (Miller and Pitnick 2002). This is likely due to benefits for females of fertilisations by high-condition males capable of producing sufficient large sperm (Lüpold et al. 2016). Such findings suggest that sperm competitiveness may often depend on the ability to successfully interact with the female environment, and that the treatment of sperm competition and cryptic female choice as independent processes probably represents a false dichotomy (Eberhard 1996; Pitnick and Hosken 2010).

### *Challenges facing study systems in post-ejaculatory sexual selection*

Clearly, many of the complexities of post-ejaculatory sexual selection remain to be resolved. Studies that attempt to address these questions face two major challenges, both related to the inherently cryptic nature of the processes involved in post-ejaculatory sexual selection. First, under conditions of sperm competition it is rarely possible to tell which male's sperm has been successful at the moment of fertilisation. Consequently, competitive fertilisation success is often inferred through paternity outcomes scored from embryos or surviving offspring, which can be influenced by a range of post-zygotic mechanisms that do not necessarily correlate with

competitive fertilisation success (Gilchrist and Partridge 1997; Olsson et al. 1999; García-González 2008a). This confounds inferences about patterns of post-ejaculatory sexual selection, which require success to be accurately assigned among sperm competitors.

The second challenge facing post-ejaculatory sexual selection studies is that in most systems it is difficult to observe the pre-fertilisation mechanisms that drive patterns of competitive success, particularly when ejaculate-ejaculate and ejaculate-female interactions are hidden within the female reproductive tract (Pitnick et al. 2009). As such, it can be difficult to pinpoint the proximate mechanisms of cryptic female choice, or measure traits of competing ejaculates *in vivo*. Without mechanistic knowledge of the male- and female-controlled processes that affect post-ejaculatory interactions, our ability to identify adaptive responses to post-ejaculatory sexual selection in both sexes is limited (Manier et al. 2010; Lüpold et al. 2013).

There is considerable interest in the development of techniques and systems that enable the direct, real-time visualisation of competitive interactions among gametes and fertilisation outcomes. There has been some recent interest in fluorescent stains that can be applied *in vitro* to ejaculated sperm, notably in a series of *in vitro* fertilisation studies using DNA fluorochromes to estimate competitive fertilisation success in the house mouse *Mus musculus* (Martín-Coello et al. 2009; Firman and Simmons 2014, 2015). Such staining techniques offer experimental flexibility in that they can be applied to the ejaculates of any male, and could thus be used across numerous competitive scenarios. However, they have yet to be widely applied in other systems, likely because *in vitro* fertilisations are logistically challenging for most internally fertilising animals and lack biologically realistic female environments. There have been exciting recent developments in two invertebrate model systems, *Drosophila melanogaster* (Manier et al. 2010; Droge-Young et al. 2012) and *Macrostomum lignano* (Marie-Orleach et al. 2014), in which genetically engineered lines of males that express fluorescent proteins in their sperm have been used to distinguish competitive outcomes of sperm from different males *in vivo*. These lines are already being applied in these systems to examine complex, realistic patterns of post-ejaculatory sexual selection in unprecedented detail (Lüpold et al. 2012, 2013; Manier et al. 2013; Marie-Orleach et al. 2016). The development of such techniques for visualising post-ejaculatory interactions across a wider range of systems could offer exciting possibilities for resolving outstanding questions in sexual selection.

### **1.3 Broadcast spawners: Darwin's neglected taxa**

#### *Importance of broadcast spawners in sexual selection research*

Despite the central importance of sexual selection across most sexually reproducing species, there has been a general taxonomic bias of research in both pre- and post-ejaculatory sexual selection. Sexual selection has predominantly been studied in complex, highly mobile animals with well-developed copulation and internal fertilisation, such as fish, terrestrial vertebrates and arthropods (Thornhill and Alcock 1983; Andersson 1994; Birkhead and Møller 1998; Simmons 2001; Shuker and Simmons 2014). Externally fertilising taxa, particularly marine invertebrates that exhibit the ancestral reproductive strategy of broadcast spawning (where sperm and eggs are released directly into the ocean), have been neglected for much of the history of sexual selection research. Darwin himself pointed to the often sessile or sedentary nature of such taxa, and their general lack of obvious sexual dimorphisms or well developed sensory systems, as evidence that they were unlikely to be targets of mating competition or mate choice (Darwin 1871). However, by overlooking broadcast spawning marine taxa, we have missed an opportunity to focus on systems where (a) sexual selection is constrained to act almost exclusively on post-ejaculatory gamete-level interactions (Evans and Sherman 2013), and (b) competitions for fertilisation are not hidden in the female reproductive tract.

It has recently been argued that because of their ancestral mode of reproduction, broadcast spawning organisms have the potential to increase our understanding of the evolutionary origins of sexual selection in 'higher order' taxa (Levitan 2010; Evans and Sherman 2013). Indeed, Parker (2014) recently presented a conceptual model suggesting that gamete interactions in ancestral external fertilisers played critical roles in the evolutionary succession of sexual reproduction (the "sexual cascade"). This model suggests that ancestral gametic interactions, including gamete competition, likely led to the transition from isogamy (gametes of equal size) to anisogamy (gametes of unequal size) (see also Lehtonen and Kokko 2011), thus generating the conditions necessary for sexual selection (asymmetrical investment in reproduction among sexes; Jennions and Kokko 2010; Kokko et al. 2012). Subsequently, synchronous broadcast spawning and conditions of intense sperm competition in early multicellular animals may have been an important selective component favouring transitions to copulation and internal fertilisation, and eventually to pre-ejaculatory sexual selection (Lehtonen and Parker 2014; Parker 2014). Therefore, the complexities of post-ejaculatory sexual selection in extant broadcast spawning systems could reveal much about evolutionary transitions in sexual selection. Moreover, given that post-

ejaculatory sexual selection remains a pervasive evolutionary force throughout most reproductive systems (Birkhead and Møller 1998), the mechanisms of gamete interactions in organisms that exhibit broadcast spawning could have direct relevance to a multitude of taxa.

#### *Patterns of post-ejaculatory sexual selection in broadcast spawners*

Despite the historical paucity of sexual selection studies on broadcast spawners, fascinating patterns of post-ejaculatory sexual selection are starting to emerge in these taxa. Reproductive success and patterns of sexual selection in both sexes are likely to vary in broadcast spawners depending on aggregation behaviour and spawning densities (Levitan 1998, 2010). When spawning individuals are sparse, sperm limitation is likely to select for increased gamete encounter rates and the formation of multi-individual spawning aggregations (Levitan et al. 1992; Levitan 2004). On the other hand, when spawning densities are high, sperm competition and the risk of polyspermy (entry of multiple sperm into eggs leading to developmental failure) are likely the dominant selective forces (Levitan 2004; Levitan and Ferrell 2006; Parker et al. 2017).

Several studies suggest that the presence of ejaculates from multiple males during spawning has benefits for females beyond overcoming sperm limitation, by providing the opportunity for post-ejaculatory mate choice for genetically desirable males (Evans and Marshall 2005; Marshall and Evans 2005; Aguirre et al. 2016). Specifically, these studies indicate that females might bias fertilisations toward sperm from more compatible males, which is consistent with evidence from non-competitive experiments that suggest male-by-female compatibility is an important component of fertilisation success and offspring survival in broadcast spawners (Evans et al. 2007, 2012; Lymbery and Evans 2013; Sherman et al. 2015). Currently, the precise compatibility benefits (e.g. inbreeding avoidance, increased offspring heterozygosity) underlying these fertilisation biases are unknown. It is also unclear how such patterns of post-ejaculatory mate choice interact with sperm competition to influence selection on male traits. However, as outlined below, broadcast spawners offer remarkably tractable systems for addressing such complex questions, which have been difficult to address in most studies of post-ejaculatory sexual selection.

The combination of relative immobility and external fertilisation means that for broadcast spawners there is limited opportunity for post-spawning control by either sex over gamete interactions. This means that in most cases the only post-ejaculatory means of influencing the outcome of sperm competition are via gamete-level mechanisms. Recent reviews have identified



two stages of sperm-egg interactions as putative mechanisms of gamete-level mate choice in broadcast spawners (Evans and Sherman 2013; Beekman et al. 2016). First, unfertilised eggs release attractant molecules that guide sperm up a chemical gradient (sperm chemoataxis; Miller 1985; Eisenbach 1999). Most research on egg chemoattractants has focused on their roles in overcoming sperm limitation (by increasing the effective target size of eggs) and maintaining reproductive barriers between species (Miller et al. 1994; Miller 1997; Jantzen et al. 2001; Riffell et al. 2004). However, recent findings of within-species variation in sperm chemotaxis in the mussel *Mytilus galloprovincialis* (Evans et al. 2012; Oliver and Evans 2014) raise the possibility that egg chemoattractants might also moderate sperm competition, although this has yet to be tested directly in competitive fertilisation trials. Second, once gametes have made contact, gamete recognition proteins on sperm and their associated receptors on eggs mediate successful fusion and fertilisation (Vacquier 1998; Swanson and Vacquier 2002). For example, studies in several sea urchin species have found that the gamete recognition protein bindin can differentially influence intraspecific fertilisation rates in response to conditions of sperm limitation and polyspermy (e.g. Levitan and Ferrell 2006), and can bias competitive fertilisations toward males with the same bindin genotype as females (Palumbi 1999). Both of these mechanisms of gamete interaction (egg chemoattractants and gamete recognition proteins) occur throughout sexually reproducing taxa beyond broadcast spawners (Miller 1985; Vacquier 1998; Clark et al. 2006; Eisenbach and Giojalas 2006), and have the potential to be widespread mediators of post-ejaculatory sexual selection (Firman and Simmons 2015).

#### **1.4 Study system: *Mytilus galloprovincialis***

The broadcast-spawning blue mussel, *Mytilus galloprovincialis*, is an ecologically dominant member of temperate sub-tidal communities (Daguin and Borsa 2000) that is emerging as a fascinating and highly tractable experimental system for the study of gamete interactions. This gonochoristic (i.e. the sexes are separated in different individuals) bivalve forms large aggregations that spawn synchronously throughout the reproductive season, and fertilisation is characterised by complex interactions among eggs and sperm of multiple individuals (e.g. Evans et al. 2012; Fitzpatrick et al. 2012; Sherman et al. 2015; Eads et al. 2016a). In *M. galloprovincialis*, egg chemoattractants have been shown to induce differential changes in sperm phenotypes (swimming direction, motility and physiology) depending on the specific male-female pairing (Evans et al. 2012; Kekäläinen and Evans 2016), effects which correspond with variation in offspring fitness among male-female crosses (Oliver and Evans 2014). This remarkable level of

individual-specific plasticity raises the intriguing possibility that differential molecular changes might regulate sperm behaviour and ejaculate-female interactions. Moreover, if differential sperm chemotaxis affects the outcomes of competition among multiple ejaculates (which has yet to be tested), it could have widespread implications for our understanding of post-ejaculatory mate choice. Sperm chemoattraction is a taxonomically ubiquitous form of gamete signalling, in both external and internal fertilisers (Miller 1985; Eisenbach and Giojalas 2006), and differential attraction of competing ejaculates could provide females with fine-scale control over which sperm fertilise their eggs.

In addition to the male-by-female interactions that characterise fertilisation in *M. galloprovincialis*, there may also be overall patterns of post-ejaculatory selection on male traits. In non-competitive fertilisation environments, which controlled for male-by-female effects by pooling eggs from multiple females, Fitzpatrick et al. (2012) reported complex patterns of non-linear selection on combinations of sperm traits, including velocity, motility and their morphology. Overall, selection favoured ejaculates with low sperm motility and velocity, and those with highly curved sperm swimming paths and small sperm heads (Fitzpatrick et al. 2012). Possible explanations for these patterns of selection include functional links between these traits and the efficiency of sperm-egg signalling and gamete encounter rates. For example, when sperm are first released in broadcast spawners, the most efficient searching pattern for detection of egg chemoattractants is to swim in circular or helical trajectories (Friedrich and Jülicher 2007). Once sperm detect a gradient of chemical signals, the chemotactic response is to swim in faster, straighter spirals toward the egg (Kaupp et al. 2006). Similarly, slower swimming sperm may be able to search for eggs over longer periods of time, which would provide an advantage under conditions of sperm limitation (as mimicked by the experimental conditions of this study) (Levitan 1998). However, typical mass spawning events in *M. galloprovincialis* are likely to result in competitive conditions with high local densities of sperm from multiple males (Wilson and Hodgkin 1967; Villalba 1995; Gosselin 2004). Studies in this species have so far lacked the capacity to assign fertilisation success when ejaculates from multiple males compete; therefore, selection under more biologically realistic conditions of sperm competition has yet to be examined.

*Mytilus galloprovincialis* offers a promising candidate system in which to overcome the difficulty of observing post-ejaculatory competitive interactions and fertilisations. A unique aspect of bivalve biology presents the opportunity to exploit a new tool for visualising the outcomes of sperm

competition. In a range of bivalve species, including *M. galloprovincialis*, embryos inherit both paternal (sperm) and maternal (egg) mitochondria through the process of doubly uniparental inheritance (DUI; Zouros 2013). Although the fate of paternal mitochondria through development ultimately differs for male and female offspring (Cao et al. 2004), immediately following fertilisation they can be easily visualised in all zygotes when stained with a vital mitochondrial dye (Obata et al. 2006). This feature of bivalve reproductive biology presents the opportunity of fluorescently labelling the mitochondria in the sperm of individual males and tracking their fertilisation success when in competition with those from rival males. This technique, when applied using *in vitro* fertilisations, offers unique opportunities to unravel the complexities of post-ejaculatory sexual selection under highly controlled and biologically realistic competitive scenarios.

### **1.5 Aims and scope of thesis**

In this thesis, I provide a detailed investigation of the gamete-level mechanisms and outcomes of post-ejaculatory sexual selection for males and females in *Mytilus galloprovincialis*, using a range of novel experimental approaches. This thesis is structured as four data chapters (Chapters Two - Five) formatted as manuscripts for publication, each focusing on a different aspect of post-ejaculatory gamete-interactions.

In Chapter Two, I develop a technique for using mitochondrial staining, as described above, in competitive *in vitro* fertilisation experiments. In this first empirical chapter I demonstrate that this technique can be used reliably to track the competitive fertilisation success of individual ejaculates, without impairing sperm function or performance. In Chapter Three, I apply this staining technique, together with a novel multi-step chemotaxis assay, to investigate whether differential male-by-female interactions, driven by egg chemoattractant gradients, allow females to bias competitive fertilisation success toward compatible ejaculates. In addition to male-by-female effects on competitive fertilisation success, I also examine overall variation among males in the competitive ability of their sperm. In Chapter Four, I explore the molecular mechanisms underlying the phenotypic effects of egg chemoattractants on sperm. Specifically, I use RNA Sequencing (RNA-Seq) technology to quantify transcript profiles of sperm with and without exposure to egg chemoattractants, and assess whether chemoattractant signals induce changes in gene expression.

In Chapter Five, I return to the question of overall variation in sperm competitiveness among males, and further capitalise on the development of the sperm dyes developed in Chapter Two to investigate whether there are patterns of sexual selection on ejaculate traits that emerge across reproductive scenarios with gametes from many males and females. In this chapter, I simulate biologically realistic multi-individual spawning events and use the mitochondrial staining technique to quantify the competitive success of each male's ejaculate under simulated multi-male spawning events. In this final empirical chapter, I quantify a number of sperm swimming parameters and morphological traits and apply statistical multivariate selection techniques to explore overall patterns of linear and non-linear selection on combinations of these ejaculate traits. Finally, in the General Discussion (Chapter Six), I summarise the findings and broad conclusions from my data chapters and provide suggestions for future research.

## CHAPTER TWO

**Fluorescent sperm offer a method for tracking the real-time success of ejaculates when they compete to fertilise eggs**

*Published in Scientific Reports*

*This chapter is presented as it appears in publication, apart from minor changes to formatting and referencing for consistency with previous and subsequent chapters.*



## 2.1 Abstract

Despite intensive research effort, many uncertainties remain in the field of gamete-level sexual selection, particularly in understanding how sperm from different males interact when competing for fertilisations. Here, we demonstrate the utility of broadcast spawning marine invertebrates for unravelling these mysteries, highlighting their mode of reproduction and, in some species, unusual patterns of mitochondrial inheritance. We present a method utilising both properties in the blue mussel, *Mytilus galloprovincialis*. In mytilids and many other bivalves, both sperm and egg mitochondria are inherited. We exploit this, using the vital mitochondrial dye MitoTracker, to track the success of sperm from individual males when they compete with those from rivals to fertilise eggs. We confirm that dying mitochondria has no adverse effects on in vitro measures of sperm motility (reflecting mitochondrial energetics) or sperm competitive fertilisation success. Therefore, we propose the technique as a powerful and logistically tractable tool for sperm competition studies. Importantly, our method allows the competitive fertilisation success of sperm from any male to be measured directly and disentangled from confounding effects of post-fertilisation embryo survival. Moreover, the mitochondrial dye has broader applications in taxa without paternal mitochondrial inheritance, for example by tracking the dynamics of competing ejaculates prior to fertilisation.

## 2.2 Introduction

Darwin (1871) first proposed sexual selection as an evolutionary force acting on variation in reproductive success caused by (1) intrasexual competition for mates (typically among males), and (2) intersexual mate choice (typically females choosing preferred males). Since then, sexual selection has become a major focus of evolutionary and behavioural research (Clutton-Brock 2007). A critical turning point in the field of sexual selection was the recognition that females often mate with multiple males, or their eggs are exposed to sperm from multiple males, meaning that sexual selection can continue after gamete release (Birkhead and Møller 1998). This occurs as sperm competition, where ejaculates from rival males compete for fertilisations (Parker 1970), and cryptic female choice, where females influence the outcome of such contests (Thornhill 1983; Eberhard 1996). Both of these mechanisms of sexual selection are widespread across most sexually reproducing taxa and constitute important evolutionary forces acting on both sexes (Simmons 2005; Pitnick et al. 2009; Simmons and Fitzpatrick 2012). Although these processes are commonly termed 'post-copulatory sexual selection' (Birkhead and Pizzari 2002), we prefer the term 'gamete-level sexual selection' to include externally fertilising animals that do not pair or copulate.

Despite intensive research on gamete-level sexual selection, there remains a taxonomic bias toward mobile, terrestrial and internally fertilising animals (Birkhead and Møller 1998; Simmons 2001; Evans and Sherman 2013). In particular, relatively few studies have focused on broadcast spawning marine invertebrates. Typically, these animals have sedentary or sessile lifestyles and both sexes release gametes directly into the ocean, where fertilisation occurs (Giese and Kanatani 1987). Although largely neglected in the context of sexual selection (but see Levitan 1998; Evans and Sherman 2013), broadcast spawners exhibit several attributes that make them ideally suited for understanding gamete-level sexual selection. First, the absence of mating competition or mate choice prior to gamete release means that sexual selection operates exclusively through gamete-level interactions (Evans and Sherman 2013). Second, broadcast spawners offer highly tractable systems for controlled *in vitro* experiments on gamete-level interactions. This tractability has been utilised in recent studies to characterise patterns of multivariate selection on gametes (Fitzpatrick et al. 2012; Johnson et al. 2013), examine variation in male-female gametic and genetic compatibilities (Evans et al. 2007; Lymbery and Evans 2013), and explore the



transmission of non-genetic paternal effects through sperm (Crean et al. 2012, 2013; Ritchie and Marshall 2013). Finally, because broadcast spawning is likely the ancestral animal reproductive strategy (Levitan 1996, 2005), the selective forces shaping this form of reproduction may yield insights into early evolutionary transitions, such as anisogamy to isogamy and external to internal fertilisation (for a recent theoretical model of this "sexual cascade" of events, see Parker 2014).

One key challenge facing researchers studying gamete-level sexual selection is to determine male reproductive success at the moment of conception. Even in external fertilisers, where gamete interactions are not hidden, there are considerable logistical challenges in identifying the outcome of sperm competition at fertilisation. Sperm competitiveness has typically been estimated through paternity analyses, which involves assigning offspring parentage among two or more putative sires using genetic markers (Simmons and Fitzpatrick 2012). Although paternity success is clearly an important component of a male's reproductive fitness, its use in understanding sperm competition potentially confounds variation in embryo viability with variation in fertilisation success (Birkhead et al. 2004; García-González and Simmons 2005; García-González 2008a). Embryo viability can be influenced by post-competition factors such as genetic sire effects (García-González and Simmons 2005; Ivy 2007), genetic compatibilities between males and females (Pitcher and Neff 2007; Lymbery and Evans 2013), maternal allocation (Nagler et al. 2000; Tregenza et al. 2003; Eads et al. 2012) and non-genetic paternal effects (Crean et al. 2013; Ritchie and Marshall 2013). Moreover, distinguishing between sperm competitive success on the one hand and offspring fitness on the other is crucial for evaluating whether processes such as 'good sperm' or 'compatible genes' underlie gamete-level sexual selection (García-González 2008a).

Here, we propose a technique for directly examining competitive fertilisation success, using the broadcast spawning blue mussel, *Mytilus galloprovincialis* (Lamarck, 1819) as a model system. *Mytilus galloprovincialis* is a sessile, broadcast spawning bivalve with several characteristics that make it an ideal putative model system for gamete-level sexual selection. Individuals form large aggregations on intertidal substrates in temperate zones and both sexes spawn synchronously during winter months, meaning sperm and eggs from

multiple individuals come into contact during each reproductive event. Moreover, unlike most animals, many bivalves (including *Mytilus* spp.) inherit mitochondrial DNA (mtDNA) from both parents in a phenomenon known as doubly uniparental inheritance (DUI; Zouros et al. 1994). While the ultimate fate of paternal and maternal mtDNA differs depending on the sex of the offspring (Breton et al. 2007), all embryos initially contain mitochondria transferred from the father's sperm (Cao et al. 2004; Obata et al. 2006). This presents the opportunity of labelling a male's sperm with a vital fluorescent mitochondrial dye, allowing these sperm to compete with (undyed) sperm from other males, and tracking the real-time competitive fertilisation success of labelled sperm by counting eggs with labelled mitochondria. We develop the protocol for using this technique in evaluating sperm competition, using the mitochondria-specific vital dye MitoTracker Green FM. We test, in paired designs, whether the mitochondrial dye has any adverse effects on sperm motility and competitive fertilisation success.

## **2.3 Methods**

### *Sampling and spawning*

We collected mussels from Cockburn, Western Australia (32°14'03.6''S, 115°76'25''E) during June to September 2014 and maintained them in aerated aquaria of recirculating seawater at the University of Western Australia until required for experiments (within 1-2 weeks of collection). Spawning was induced using a temperature increase from ambient to 28 °C (Evans et al. 2012; Fitzpatrick et al. 2012; Oliver and Evans 2014). Once an individual began spawning and its sex was determined, it was immediately removed from the water bath, washed in filtered seawater (FSW) to prevent contamination of gametes, placed in an individual 250 mL plastic cup and covered with FSW. Following spawning, egg densities were estimated by counting the number of eggs in a known volume under a dissecting microscope, and sperm densities were estimated from subsamples of sperm (fixed using 1% formalin) using an improved Neubauer haemocytometer. Gametes were then diluted to the concentrations required for trials (see below).

### *Mitochondrial dye application*

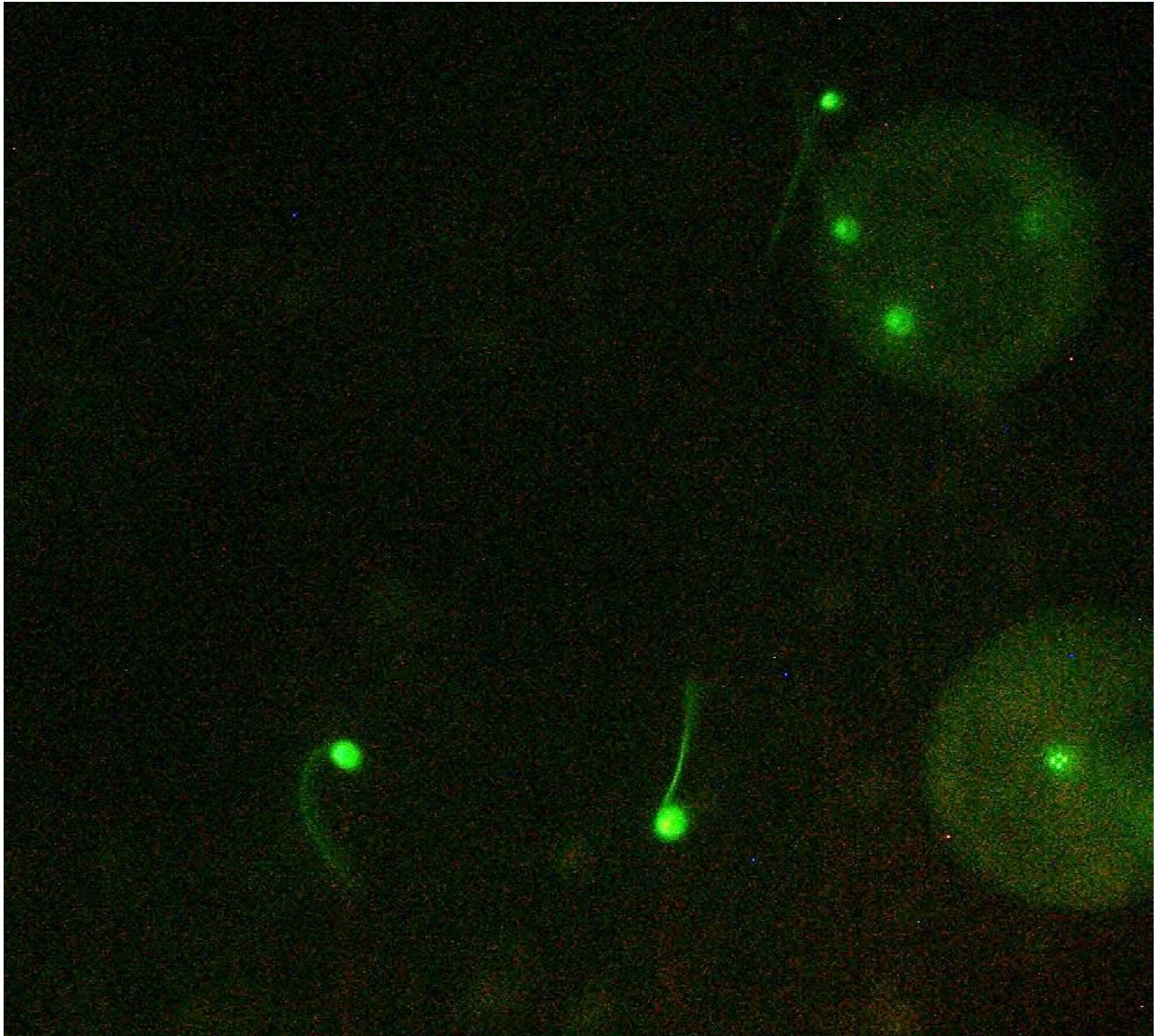
We used the mitochondria-specific vital dye MitoTracker Green FM (Molecular Probes, Eugene, OR, USA) to stain sperm mitochondria (Fig 2.1). We initially trialled a second dye

colour (MitoTracker Red) but this proved to be unreliable in terms of consistency of uptake (no motile sperm were visibly labelled under fluorescence). Hence our competitive experiments involved reciprocally dyeing each competing male's sperm green, rather than labelling different males' sperm with different colours (see below). MitoTracker Green has been used previously to stain mitochondria of sperm in *Mytilus* spp. and other bivalves in order to follow paternal mitochondria through development (Cao et al. 2004; Obata and Komaru 2005; Milani et al. 2011). We followed a protocol adapted from these studies for staining sperm in our experiments. All samples and solutions of dye were kept in the dark. Stock solutions of 1 mM dye were created by suspending 50 µg of MitoTracker Green in 74.5 µL dimethyl sulfoxide (DMSO). These were diluted with FSW to 10 µM working solutions. Sperm were stained in 1 mL samples (see below for sperm concentrations) containing 50 µL of working dye solution, i.e. 500 nM concentration of MitoTracker Green. Stained sperm were incubated in the dark for 10 minutes at room temperature. This was sufficient for uptake of dye by all cells in the sample (preliminary observations), while minimising sperm ageing effects, which are known to influence fertilisation rates in *M. galloprovincialis* (Fitzpatrick et al. 2012).

#### *Measuring sperm motility traits of dyed and undyed sperm samples*

Our first experiment compared the motility (swimming characteristics) of dyed and undyed sperm samples. We prepared two 950 µL subsamples of sperm at  $5 \times 10^6$  sperm mL<sup>-1</sup> from each of n = 18 males; 50 µL of MitoTracker working solution was added to one subsample, and 50 µL of FSW to the other. After incubation of subsamples (see above), we placed 5 µL in a 12-cell multi-test slide, previously washed with 1% polyvinyl alcohol to avoid sperm sticking to the slide. Sperm motility was characterised using computer-assisted sperm analysis (CASA; Hamilton-Thorne CEROS, Beverly, MA, USA). We used threshold values for defining static cells of 19.9 µm/s VAP and 4 µm/s for VSL. For half the males, we measured undyed samples first, while in the other half, we measured dyed samples first. A mean of  $149 \pm 11.7$  s.e.m. motile sperm were recorded per sample. We calculated the percentage of motile sperm from the motile and total cell counts. We measured the following seven sperm motility parameters of the motile sperm, which are commonly used in studies of sperm competition and have high within-sample repeatability in *M. galloprovincialis* (Fitzpatrick et al. 2012): average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL),

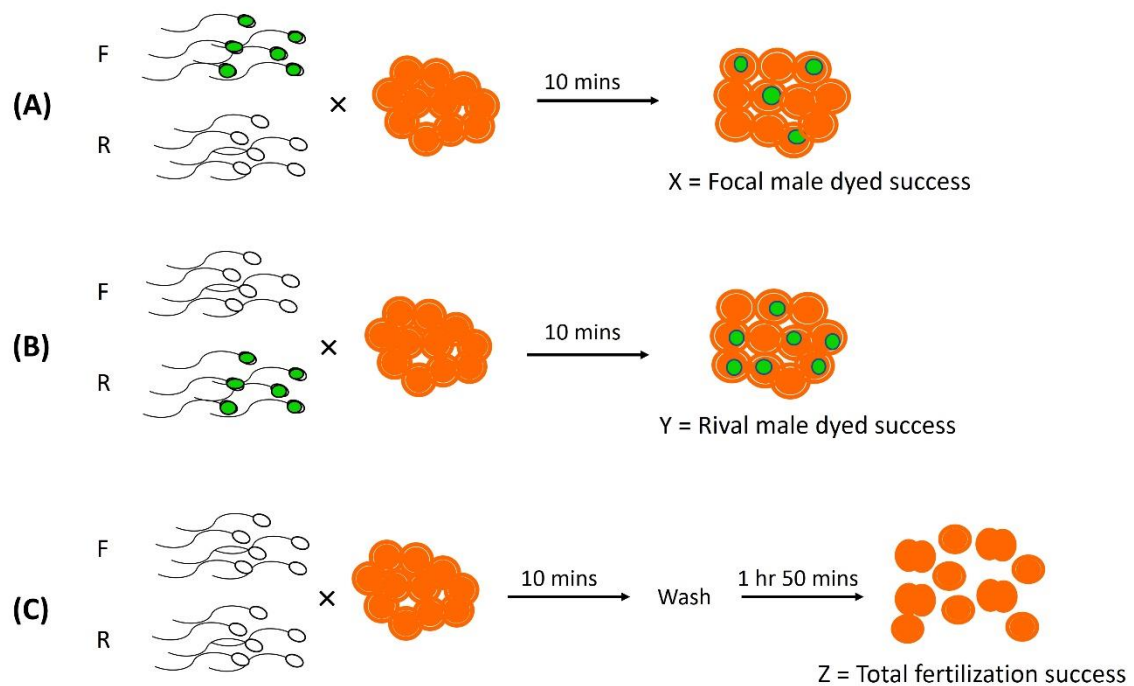
straightness (STR), linearity (LIN), beat cross frequency (BCF), the amplitude of lateral head displacement (ALH).



**Figure 2.1.** *Mytilus galloprovincialis* sperm labelled with MitoTracker Green and fertilised eggs containing mitochondria from labelled sperm. Viewed using a Zeiss Axio Imager A1 fluorescent microscope, image captured using AxioCam MRc5 and Axiovision software. Image brightness and contrast adjusted using ImageJ software.

### *Competitive fertilisation trials*

To compare competitive fertilisation success of dyed and undyed sperm from the same males, we set up pairs of reciprocal competitive fertilisation trials in which sperm from the same two males, one arbitrarily chosen as the 'focal' male and the other as his 'rival', competed for fertilisation of a single female's eggs. In these trials, 1 mL samples of sperm from each male at concentrations of  $1 \times 10^5$  cells mL<sup>-1</sup> were added to 2 mL of eggs at  $1 \times 10^4$  cells mL<sup>-1</sup>; i.e. a final sperm:egg ratio of 10:1, shown in previous studies in this species (Oliver and Evans 2014) to avoid 0% fertilisation and ceiling effects (lower variation in fertilisation rates than expected; Levitan and Petersen 1995; Fitzpatrick et al. 2012). In each fertilisation, only one male's sperm was dyed; in (A), the focal male's sperm was dyed, and in (B) the rival male's sperm was dyed (Fig. 2.2). We then estimated competitive fertilisation success of the dyed sperm in each context as the proportion of eggs containing labelled mitochondria (e.g. see labelled eggs in Fig. 2.1). If we denote the focal male's dyed success as X (from trial A) and his rival's success as Y (from trial B), we expect  $X = 1 - Y$  if (i) the dye has no effect on sperm competition success, and (ii) in every trial all eggs were fertilised. We could not meet the second assumption, however, because raising sperm concentrations to levels that resulted in 100% fertilisation would have risked polyspermy, resulting in zygote failure (Dufresne-Dubé et al. 1983), and ceiling effects. We therefore conducted a third concurrent competitive cross (C), involving undyed sperm from the same two males to determine overall sperm fertilisation success, Z. With this estimate we set up a paired comparison in which, under a null hypothesis of no effect of dye, we expect  $X = Z - Y$  (Fig. 2.2). Ten paired comparisons (n = 20 males, 10 females) were used in this experiment. For further detail regarding the fertilisation procedures, see the Supplementary Methods.



**Figure 2.2.** Experimental design for test of the effect of MitoTracker Green on competitive fertilisation success. Each paired comparison involved three crosses between sperm from the same two competing males (focal and rival) and eggs from the same female. (A) Focal male's sperm dyed to estimate  $X$ ; (B) rival male's sperm dyed to estimate  $Y$ ; (C) both males' sperm undyed and overall fertilisation rate,  $Z$ , measured. The focal male's dyed success,  $X$ , was then compared to his undyed success, estimated as  $Z-Y$ .

### Data analyses

Statistical analyses were carried out in R version 3.1.2 (R Core Team 2017). For the sperm motility experiment, all sperm traits met the assumption of normality of differences between dyed and undyed values (Shapiro-Wilk tests,  $P > 0.05$ ), except BCF ( $W = 0.89$ ,  $P = 0.038$ ). Measures of BCF were therefore square root transformed before performing further analyses (after transformation:  $W = 0.91$ ,  $P = 0.083$ ). The percentage of motile sperm in dyed and undyed samples was compared using a paired  $t$ -test. To compare the sperm motility traits of males across dyed and undyed samples, we reduced the highly correlated traits to principal components (PCs) and used PC scores in  $t$ -tests. Specifically, we calculated the differences between trait scores of undyed and dyed sperm samples for each male and each trait, then performed a principal component analysis on the differences using the

package 'FactoMineR' (Husson et al. 2015) from which we retained PCs with eigenvalues >1. The PC scores for males were used as sets of differences between undyed and dyed samples and tested with one-sample *t*-tests ( $H_0: \mu = 0$ ).

Dyed (X) and undyed (Z-Y) competitive fertilisation success estimates were also compared using paired analyses. Competitive fertilisation success, however, was a binomial response variable (i.e. proportions with denominator Z, the overall fertilisation rate). These data were modelled using a generalised linear mixed-effects model (GLMM) with a logit link function in the R package 'lme4' (Bates et al. 2014). The model was fit and parameters estimated using the Laplace approximation of the log-likelihood (Raudenbush et al. 2000). The model included the fixed effect of dye (i.e. dyed or undyed estimate) and a random effect for pair. The significance of the fixed effect was estimated using a Wald *t*-test, recommended by Bolker et al. (2008) to account for uncertainty in overdispersion estimates, because our GLMM was overdispersed (residual deviance 53.18 on 17 degrees of freedom, dispersion parameter = 3.13). Overdispersion in mixed-effects models can also be accounted for by adding an observation-level random effect, with a separate level for each individual measurement (Browne et al. 2005). In this case, adding an observation-level random effect resulted in underdispersion (residual deviance 2.83 on 17 degrees of freedom, dispersion parameter = 0.18), but did not change the conclusions regarding the fixed effect (Wald  $Z = -0.09$ ,  $P = 0.962$ , compare to test on original model in Results).

We conducted simulations to determine our power in the competitive fertilisation success experiment - i.e. the smallest difference in competitive fertilisation success between dyed and undyed samples that we could have detected with a power of 0.8 or more, given our sample size and the variation in our dataset. We provide a detailed procedure for the simulations in the Supplementary Methods. Briefly, in each simulation we sampled 10 sets of paired values (dyed and undyed treatments) from binomial distributions in which a male's sperm had a specified decrease in probability of fertilisation success when dyed. We then modelled these using a GLMM as described. We performed 1000 simulations for each specified difference in probability of success, and calculated the power of detecting that difference as the proportion of significant *P*-values out of 1000.

## 2.4 Results

### *Determining the effect of mitochondrial dye on in vitro measures of sperm motility*

The total percentage of motile sperm was not significantly different between dyed and undyed samples (paired t-test,  $t_{17} = -0.62$ ,  $P = 0.54$ ). We estimated seven motility traits from the motile sperm (Table 2.1), calculated the difference in each trait between dyed and undyed samples of each male, then reduced the set of differences in traits to principle components (PCs; see Methods). Two PCs with eigenvalues  $>1$  (collectively accounting for 88.87% of the variance in trait differences) were retained for the analysis. The first PC was loaded positively by the differences in VAP, VSL and LIN, and negatively by the difference in BCF, while the second PC was loaded positively by the differences in VCL and ALH and negatively by the difference in STR (Table 2.1). The means of the males' scores for each PC (representing the composite difference between dyed and undyed measures for each male) were not significantly different from zero (PC1:  $t_{17} = 58.66 \times 10^{-16}$ ,  $P = 1$ ; PC2:  $t_{17} = 6.72 \times 10^{-16}$ ,  $P = 1$ ), thus confirming no discernible effect of the sperm dye technique on the *in vitro* measures of sperm motility.

**Table 2.1.** Sperm motility traits and principle components generated from the sets of differences in values between dyed and undyed sperm samples for each trait. Shown are trait loadings, eigenvalues and cumulative per cent of variance in composite trait variables of the first two principle components.

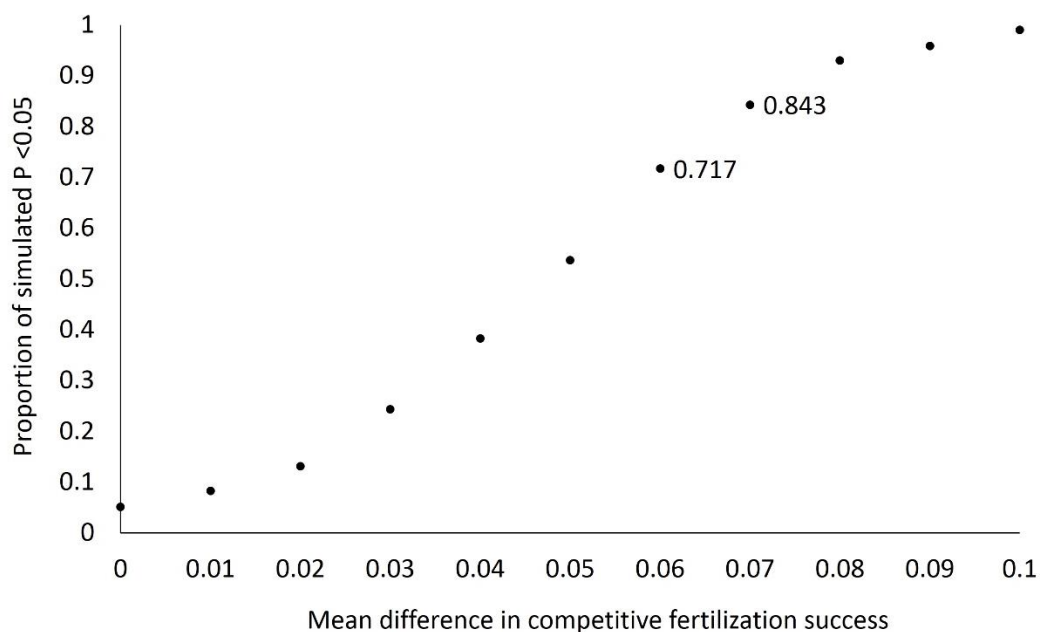
<b>Trait (difference between scores of undyed and dyed samples)</b>	<b>PC1</b>	<b>PC2</b>
VAP: average path velocity	0.94	0.28
VCL: curvilinear velocity	0.57	0.80
VSL: straight-line velocity	0.98	0.04
STR: straightness	0.50	-0.73
LIN: linearity	0.85	-0.52
ALH: amplitude of lateral head displacement	0.12	0.87
BCF: beat cross frequency*	-0.89	0.07
Eigenvalue	3.93	2.29
Cumulative per cent of variance explained	56.15	88.87

\* BCF was square root transformed prior to analyses



### Competitive fertilisations

The overall fertilisation rate (overall proportion of fertilised eggs) of each competitive cross ranged from 0.460 – 0.940 (mean =  $0.733 \pm 0.050$  s.e.m.). There was no significant difference in the probability of successful competitive fertilisations between dyed and undyed samples (Wald  $t_{17} = 0.23$ ,  $P = 0.821$ ). As there were fewer replicate pairs than in the sperm motility trials, we conducted simulations to determine the power of our experimental design to detect differences in competitive fertilisation success between dyed and undyed sperm. These revealed we had 80% power to detect a difference in the proportion of fertilisation success of between 0.06 and 0.07 (Fig. 2.3).



**Figure 2.3.** Proportion of significant simulated tests of varying differences in competitive fertilisation success between stained and unstained sperm (based on the variation and sample size in our dataset); i.e. power to detect a difference. The horizontal axis shows the varying simulated differences in the probability of success due to staining, and the vertical axis shows the proportion of significant  $P$  values from 1000 simulated tests. A power of 0.8 falls between a difference of 0.06 and 0.07.

A mean difference of 0.06 was detected as significant in 72% of simulations, and a mean difference of 0.07 in 84% of simulations. These findings indicate that we had good power to detect small differences in fertilisation success due to the dye treatment. Indeed, in our observed data the actual mean difference in fertilisation success between dyed and undyed samples was close to zero (mean difference  $-0.004 \pm 0.057$  s.e.m.; range  $-0.305 - 0.310$ )

## 2.5 Discussion

Our method for visualising the real-time success of sperm when they compete to fertilise eggs offers a tractable and potentially powerful tool for studying gamete-level sexual selection. Importantly, the MitoTracker Green mitochondrial dye had no detrimental effect on sperm behaviour and had no significant influence on the capacity of sperm to fertilise eggs when in competition with rival male ejaculates. Thus, the MitoTracker dye offers an effective and reliable method for visualising the outcome of sperm competition, with important benefits for research on gamete-level sexual selection. In particular, our proposed methods overcome a major hurdle in gamete-level sexual selection research, where success in sperm competition can typically only be inferred from offspring paternity assignment.

Our experimental confirmation that the MitoTracker dye had no discernible detrimental effects on patterns of sperm motility suggests that the dye does not disrupt sperm performance or mitochondrial function. Sperm motility traits have been linked to adenosine triphosphate (ATP) production in the sperm mitochondria (Perchee et al. 1995; Froman and Feltmann 1998), or to the size of the sperm midpiece where mitochondria are located (Firman and Simmons 2010). Furthermore, both sperm ATP content (Tourmente et al. 2013) and midpiece size (Anderson and Dixson 2002) can vary with the level of sperm competition. Importantly, the motility traits we measured can have fitness implications for males during competitive and non-competitive fertilisations. For example, numerous studies have reported a positive association between sperm velocity and fertilisation success across a range of taxa (reviewed in Simmons and Fitzpatrick 2012), although there are exceptions where slower sperm have been associated with greater fertilisation benefits (e.g. Dziminski et al. 2009; Lüpold et al. 2012). In *M. galloprovincialis*, males with slower sperm that swim in more pronounced curved paths are the most successful during non-competitive fertilisation

trials (Fitzpatrick et al. 2012). This may reflect their capacity to search for eggs, or the chemical attractants released by eggs (Kaupp et al. 2006), in a marine environment. Given our finding that the MitoTracker Green dye had no observable effect on these specific motility traits, we propose that it may be used to assess male reproductive fitness in future studies.

Consistent with the sperm motility results, we found that the mitochondrial dye did not significantly reduce competitive fertilisation success. Although there were fewer replicate pairs for this experiment than the sperm motility trials, our analyses had the power to detect small changes (proportional change of 0.06-0.07) in fertilisation success. This further suggests that the MitoTracker dye can be applied in a sperm competition context and provides a simple and cost-effective method for assessing competitive fertilisation success, negating the need to use offspring paternity assignment as a proxy for competitive fertilisation success. This is an important methodological advance because paternity success can be influenced by a range of factors operating after fertilisation, which may or may not be related to sperm competitive ability (García-González 2008a). For example, in the sea urchin *Heliocidaris erythrogramma*, variance in embryo viability and fertilisation rates are uncorrelated within male-female pairings, suggesting that fertilisation rates cannot be inferred through variance in egg hatching rates (Evans et al. 2007). In such systems, it is critical to use techniques that can directly estimate success at the point of fertilisation, such as the MitoTracker dye.

Previous studies using fluorescent dyes to distinguish competing sperm in fertility assays have mainly focused on domestic mammals. For example, dyes have been used to visualise the number of sperm from different males bound to bovine (Miller et al. 1998; Braundmeier et al. 2002) and feline (Niu et al. 2006) eggs. However, these prior studies could not directly determine which of the bound sperm actually achieves fertilisation. By contrast, the present technique enables us to track the real-time success of individual sperm as they fertilise eggs. As such, our proposed method offers a potentially powerful tool in the context of understanding the dynamics of sperm competition. Other studies have overcome the challenge of identifying sperm from individual males through the use of selected genetic lines that express green fluorescent protein in all cell types, as for example in sperm of

*Drosophila melanogaster* (Manier et al. 2010; Droge-Young et al. 2012; Lüpold et al. 2012) and all cell types of *Macrostomum lignano* (Marie-Orleach et al. 2014). As with our study, these techniques make it possible to track the real-time success of sperm, although the logistical constraints of applying such methods to internal fertilisers mean that tracking sperm competition success *in vivo* is challenging in such systems. Moreover, the present technique does not require genetically modified lines for implementation, meaning that it can be applied to the sperm of any male, including those from natural populations.

The MitoTracker technique has broad applications not only across reproductive scenarios, but also potentially across taxonomic groups. The technique allows competitive fertilisation success to be tracked in any species with DUI, which include many species where knowledge about reproductive biology has potential commercial importance to fisheries (Breton et al. 2007). Furthermore, we envisage that the mitochondrial dye technique has applications more broadly in taxa that do not have DUI of mtDNA. The mitochondrial dye could be used to track interactions of competing ejaculates *in vitro* by determining whether and how the presence of rival sperm influences pre-fertilisation performance, and examining if the capacity to influence rival sperm varies among males. For example, the use of selected lines expressing fluorescent proteins have revealed sperm displacement from the female reproductive tract by rival sperm in *M. lignano* (Marie-Orleach et al. 2014), and the adjustment of sperm swimming speed to match rival sperm in *D. melanogaster* (Lüpold et al. 2012). We suggest that dyes such as MitoTracker could be used to explore such pre-fertilisation interactions in species where it is not possible to create selected genetic lines. To our knowledge, no studies have used mitochondrial dyes in this way to track interactions between competing sperm in an evolutionary context. We note that it may be necessary to test for the absence of an effect of dye on sperm performance in different taxa, although our expectation will be that the dye can reliably be used to stain sperm in other species. We look forward to the new insights that the implementation of this technique will bring to the field of sexual selection.

## **2.6 Acknowledgements**

We thank Clelia Gasparini for help in developing the protocol for application of MitoTracker, Ashley Lymbery and Hannah Ashe for practical assistance, Angela Eads, Jukka Kekäläinen

and Cameron Duggin for assistance with collections, and Alan Lymbery and Samuel Lymbery for helpful comments on the manuscript. Funding was provided by the UWA School of Animal Biology (RAL) and the Australian Research Council (JPE). RAL was supported by the Hackett Postgraduate Research Scholarship and the Bruce and Betty Green Postgraduate Research Top-Up Scholarship.

## 2.7 Supplementary Materials

### Supplementary Methods

#### *Experimental procedures for competitive fertilisation trials*

For competitive fertilisations (A) and (B) (see Methods for experimental design), 1 mL concentrated aliquots of sperm ( $1 \times 10^7$  cells mL<sup>-1</sup>) were made up for each male. After dyeing and incubation and prior to competing dyed and undyed sperm together, excess MitoTracker was removed by centrifugation in an Eppendorf MiniSpin at 1677 g (rotor radius = 6 cm, speed = 5000 rpm) for 1 minute (spinning sperm at relatively high speeds for short periods minimises oxidative stress; Shekarriz et al. 1995; Carvajal et al. 2004), removal of supernatant, and resuspension in 1 mL FSW. Importantly, the undyed samples were treated to the same centrifugation procedure as the dyed samples. Following resuspension, we adjusted sperm samples to 1 mL of  $1 \times 10^5$  cells mL<sup>-1</sup>. The samples from the two males were then mixed together with 2 mL of eggs at  $1 \times 10^4$  cells mL<sup>-1</sup> (i.e. a final sperm:egg ratio of 10:1). Competitive fertilisations were allowed to proceed for 10 minutes in the dark (sperm mitochondria are clustered in the zygote and easily visible 10 minutes after fertilisation; Obata and Komaru 2005). We then observed samples under a Zeiss Axio Imager A1 fluorescent microscope and assayed a haphazard sample of 100 eggs, counting the number containing dyed mitochondria.

For competitive fertilisation (C), in which no sperm were dyed, we relied on traditional methods to assay fertilisation rates, i.e. counting the proportion of eggs undergoing cleavage (Marshall et al. 2000). For these trials, we implemented strict experimental controls to ensure that fertilisation conditions (other than the absence of dyes) were similar to trials (A) and (B). Aliquots of sperm from both males were treated to the same experimental procedure as previously, including centrifugation, resuspension and dilution, and added to 2 mL of eggs at the concentrations outlined above. However, we were constrained to estimate fertilisation success 2 hours after sperm were added to the eggs, when cleavage of cells is apparent, and not at 10 minutes as in trials (A) and (B). We therefore halted fertilisations at 10 minutes so that fertilisation rates used to estimate Z were comparable to those involving dyed sperm in each replicate. To achieve this, 10 minutes after sperm were added to the eggs, eggs were rinsed through a filter and retained

in new FSW. The filter pore size was 30  $\mu\text{m}$ , which retained the eggs but is many times larger than the head of *M. galloprovincialis* sperm (mean  $\pm$  s.e.m. head length =  $2.71 \pm 0.01$   $\mu\text{m}$ , mean  $\pm$  s.e.m. head width =  $2.04 \pm 0.01$   $\mu\text{m}$ ; unpublished data from Fitzpatrick et al. 2012). In preliminary trials, we did not detect any sperm cells remaining with retained eggs, or any visible damage to eggs. Fertilisation rates for these crosses remained high throughout the experiment. After developing in sperm-free water for a further 1 hour 50 minutes (total 2 hours), 1 mL of eggs were fixed in 10% buffered formalin until required to assess fertilisation rates. We assayed a haphazard sample of 100 eggs and calculated the proportion undergoing cleavage.

*Simulation procedure for determining power to detect differences in probability of competitive fertilisation with generalised linear mixed-effects model.*

We used a simulation-based approach to determine the difference in probability of successful competitive fertilisations with dyed vs. undyed sperm that we could have detected as significant with a power of 0.8 or more, given the structure of our data. The simulations used random binomial sampling in R, which requires three inputs: the number of samples ('n'), the number of trials in each sample ('size'), and the probability of success for each trial ('prob'). The output is number of successes in each sample. First, we simulated the number of undyed successful competitive fertilisations for each focal male, where 'n' = 10 focal males, and the 'size' input was the vector of actual number of trials for each focal male (corresponding to the total number of eggs fertilised across both competitors, Z; see Methods). The input for 'prob' was a vector of proportion of actual undyed competitive fertilisations  $[(Z-Y)/Z]$ ; see Methods] for each focal male, i.e. we used the observed undyed competitive sperm success of focal males to set their 'baseline' probability of success in random sampling. The random binomial sampling then used this baseline probability to provide a simulated number of undyed successful competitive fertilisations for each focal male.

We then simulated the number of dyed successful competitive fertilisations in a similar way. The inputs for 'n' and 'size' were the same as above. The input for 'prob' again used the vector of 'baseline' probabilities, but subtracted a constant effect on probability due to dye that we wanted to simulate. For example, when simulating a decrease in the probability of

success due to dye of 0.05 (5%), 'prob' was the vector of baseline probabilities minus 0.05. In this way we were able to simulate paired values of undyed and dyed success for each male. We combined the simulated undyed and dyed data, and assigned the factors 'Treatment' (dyed or undyed) and 'Pair'. We then ran a generalised linear mixed model on the simulated data (see Methods) and stored the *P*-value for the fixed effect 'Treatment'. The entire simulation procedure was repeated 1000 times for a particular simulated effect of dye, and the power to detect the simulated effect estimated by the proportion of *P*-values that were statistically significant ( $<0.05$ ).



## CHAPTER THREE

### **Egg chemoattractants moderate intraspecific sperm competition**

*Published in Evolution Letters*

*This chapter is presented as it appears in publication, apart from minor changes to formatting and referencing for consistency with previous and subsequent chapters.*



### **3.1 Abstract**

Interactions among eggs and sperm are often assumed to generate intraspecific variation in reproductive fitness, but the specific gamete-level mechanisms underlying competitive fertilisation success remain elusive in most species. Sperm chemotaxis – the attraction of sperm by egg-derived chemicals - is a ubiquitous form of gamete signalling, occurring throughout the animal and plant kingdoms. The chemical cues released by eggs are known to act at the interspecific level (e.g. facilitating species recognition), but recent studies have suggested that they could have roles at the intraspecific level by moderating sperm competition. Here, we exploit the experimental tractability of a broadcast spawning marine invertebrate to test this putative mechanism of gamete-level sexual selection. We use a fluorescently labelled mitochondrial dye in mussels to track the real-time success of sperm as they compete to fertilise eggs, and provide the first direct evidence in any species that competitive fertilisation success is moderated by differential sperm chemotaxis.

Furthermore, our data are consistent with the idea that egg chemoattractants selectively attract ejaculates from genetically compatible males, based on relationships inferred from both nuclear and mitochondrial genetic markers. These findings for a species that exhibits the ancestral reproductive strategy of broadcast spawning have important implications for the numerous species that also rely on egg chemoattractants to attract sperm, including humans, and have potentially important implications for our understanding of the evolutionary cascade of sexual selection.

### 3.2 Introduction

Sexual selection, which acts on variation in traits that influence reproductive success, almost certainly began in the sea with externally fertilising organisms (Levitan 2010; Parker 2014). In these systems, before the evolution of advanced mobility and sensory structures, there would have been limited opportunity for mating competition or mate choice prior to gamete release. Instead, synchronous broadcast spawning (where gametes from both sexes are expelled externally) and the co-occurrence of gametes from multiple individuals likely fuelled sexual selection in the form of sperm competition (competition for fertilisation among ejaculates from multiple males; Parker 1970) and cryptic female choice (biasing of fertilisation by females or their eggs toward particular ejaculates; Thornhill 1983; Eberhard 1996). Recent theory suggests that these ancestral processes of sexual selection instigated the evolutionary cascade toward many derived features of animal reproductive systems, including sexual dimorphism, internal fertilisation and precopulatory sexual selection (Parker 2014). However, sperm competition and cryptic female choice have themselves remained pervasive forms of sexual selection in most sexually reproducing taxa (Pitnick and Hosken 2010). There is, therefore, considerable empirical value in studying gamete-level interactions in extant broadcast spawners as they may provide clues into the mechanisms underlying sperm-egg interactions in a broad range of taxonomic groups (Levitan 2010; Evans and Sherman 2013).

A key goal in reproductive and evolutionary biology is to seek mechanistic insights into the processes that generate fertilisation biases during sperm competition, and in particular into the role that females play in moderating this competition (Pitnick et al. 2009; Pitnick and Hosken 2010; Firman et al. 2017). While evidence for female control over fertilisation is now compelling in many systems (e.g. Clark et al. 1999; Nilsson et al. 2003; Pilastro et al. 2004; Lovlie et al. 2013; Young et al. 2013; Firman and Simmons 2015), direct demonstrations of the underlying mechanisms remain largely elusive (but see Gasparini and Pilastro 2011; Alonzo et al. 2016). Broadcast spawning taxa offer particularly amenable and experimentally tractable systems with which to identify such mechanisms (Evans and Sherman 2013). Unlike internal fertilisers, in broadcast spawners the interactions between gametes are not hidden from view within the female reproductive tract, making it possible to visualise processes (e.g. gamete selection) that would otherwise have to be inferred indirectly. For

example, eggs of broadcast spawners can moderate the recognition and fusion of sperm at the gamete surface (Palumbi 1999; Levitan and Ferrell 2006), or select specific sperm nuclei when multiple sperm penetrate the egg (Carré and Sardet 1984). However, eggs can also influence sperm remotely (i.e. prior to the meeting of gametes) through the release of chemical attractants. This process, which is known as sperm chemotaxis, is often crucial in broadcast spawners for ensuring eggs are found and fertilised by conspecific sperm (Miller et al. 1994; Riffell et al. 2004). Moreover, it has been argued that when ejaculates from multiple conspecific males are present, such remote signalling between eggs and sperm could be an important mediator of competitive fertilisation success (Evans et al. 2012).

Although sperm chemotaxis is taxonomically widespread in both external and internal fertilisers (Miller 1985; Eisenbach 1999; Eisenbach and Giojalas 2006), its putative role in gamete-level sexual selection has only recently come to light. For example, recent studies on the broadcast spawning mussel *Mytilus galloprovincialis* have revealed that chemoattractants have differential effects on the swimming behaviour (chemotactic responses, swimming trajectory and speed; Evans et al. 2012; Oliver and Evans 2014) and physiology (acrosome reaction; Kekäläinen and Evans 2016) of sperm from different conspecific males. The strength of these effects correlate with differences in offspring survival among male-female crosses (Oliver and Evans 2014). These findings suggest that chemoattractants could promote fertilisations by genetically compatible sperm, but this has yet to be investigated under conditions of sperm competition. Moreover, the molecular processes underlying potential genetic compatibility effects are unknown. For example, differential sperm chemotaxis may be driven by gamete-level mechanisms that promote optimal levels of general offspring heterozygosity, which is often cited as an explanation of compatibility-based gamete choice (Firman et al. 2017). Alternatively, more specific patterns of genetic compatibility may apply in *M. galloprovincialis* populations, which typically contain multiple mitochondrial DNA lineages as a result of historical migration patterns (Westfall and Gardner 2010; Dias et al. 2014). What is clear, however, is that the intraspecific effects of chemoattractants on fertilisation have important fitness implications for both males and females in this system.

In this study we test whether differential sperm chemotaxis moderates gamete-level mate choice in *M. galloprovincialis*, and whether fertilisation biases attributable to differential chemotactic responses reflect underlying patterns of genetic complementarity. Our experimental design allows us to measure competitive fertilisation success directly, rather than the more usual method of estimating fertilisation success indirectly from a male's paternity share. The latter method (paternity share) can be confounded by post-fertilisation effects on offspring viability that may not be related to sperm competitiveness (García-González 2008a; García-González and Evans 2011). Here, we overcome this problem using a fluorescent dye to label the mitochondria of sperm of competing males (Chapter 2). In *M. galloprovincialis* and many other bivalves, embryos inherit both paternal and maternal mitochondria through a process termed doubly uniparental inheritance (DUI) (Zouros et al. 1994; Obata et al. 2006; Breton et al. 2007). In DUI, maternal mitochondria are inherited in the somatic tissue of all offspring, while the paternal mitochondria are ultimately transmitted to the germ line of male offspring (Breton et al. 2007). Initially, however, sperm mitochondria are transferred into all fertilised eggs (Obata et al. 2006). This feature of bivalve reproductive biology enables us to label sperm with a fluorescent mitochondrial vital dye and track their success during fertilisation when labelled sperm from focal males compete with unlabelled rival ejaculates (Chapter 2).

The primary aim of our study was to determine whether chemoattractants moderate competitive fertilisation success in *M. galloprovincialis*. To test this, we used a novel multi-step experimental protocol involving multiple 2 x 2 factorial crosses to determine whether egg chemoattractants moderate the success of ejaculates when they compete to fertilise eggs (see Methods). We also tested whether fertilisation biases induced by egg chemoattractants (ECs) reflect patterns of genetic complementarity between focal sperm competitors and female EC donors. Our highly controlled design enabled us to: (1) directly examine variation in competitive fertilisation success using sperm dyes, therefore controlling for post-fertilisation effects on embryo viability; (2) separate the effects of males, females and their interactions on competitive fertilisation success; and (3) isolate the effect of differential chemical attraction as the female-moderated mechanism for biasing competitive fertilisations. Importantly, our design controls for stochastic variation in fertilisation that could be caused by random sampling of rival males, by using sperm from a

standard rival to compete with the dyed sperm of focal males within each factorial (García-González 2008b; García-González and Evans 2011). Our ensuing results provide the first direct evidence in any system that differential attraction of sperm up an egg chemoattractant gradient moderates intraspecific competitive fertilisation success. Furthermore, we find that fertilisation biases induced by egg chemoattractants reflect both preferences for unrelated males at nuclear loci and the selection of the same mitochondrial DNA lineage, thus revealing the putative genetic benefits of gamete-level mate choice in this system.

### **3.3 Methods**

#### *Study species and spawning*

*Mytilus galloprovincialis* is a sessile, gonochoristic bivalve mollusc that forms large aggregations on intertidal substrates in temperate regions of both Hemispheres. *Mytilus galloprovincialis* is distributed across the southern coast of Australia (Westfall and Gardner 2010), with phylogenetic studies indicating that populations contain signatures of both a native Southern Hemisphere lineage and a more recent introduction of Northern Hemisphere individuals (Westfall and Gardner 2010; Colgan and Middelfart 2011; Dias et al. 2014). Nevertheless, there appears to have been extensive reproductive mixture of individuals from these different lineages in Australian populations (Westfall and Gardner 2013). We collected mussels from Woodman Point, Cockburn, Western Australia (32°14' 03.6"S, 115°76' 25"E) during the 2015 spawning season (June-September), and maintained them in aquaria of recirculating seawater at the University of Western Australia until required (within one week of collection). Spawning was induced using a temperature increase from ambient to 28°C (Chapter 2). Once an individual began spawning and its sex was determined, we immediately removed it from the spawning tank, washed it in filtered seawater (FSW) to remove possible contaminating gametes, placed it in an individual 250 mL cup and covered it in FSW. Once gametes were suitably dense, we removed the spawning individuals, estimated egg concentration by counting the number of cells in a homogenised 5 µL sample under a dissecting microscope, and estimated sperm concentration from subsamples (fixed in 1% formalin) using an improved Neubauer haemocytometer. We used these estimates to dilute gametes to their required concentrations for ensuing trials (see below).

### *Experimental overview*

We used a multi-step cross-classified design with blocks of two focal males (M1 and M2) and two focal females (F1 and F2) (Fig. 3.1A; the steps involved in a trial from a single cell of the block are shown in Fig. 3.1B). The initial steps involved differential sperm chemotaxis assays, where sperm from each focal male (dyed sperm, see below) competed with undyed sperm from a standard rival (SR) male in the presence of a chemoattractant gradient from each of the two focal females (EC1 and EC2). Therefore, four competitions were performed per block; M1 vs. SR in EC1, M1 vs. SR in EC2, M2 vs. SR in EC1, and M2 vs. SR in EC2. The final step involved competitive fertilisation assays, where eggs from a single standard female (different to the focal females used for chemoattractant gradients) were used to assess the competitive fertilisation success of the focal male (in competition with the standard rival) in each cross. This latter step enabled us to attribute differences in competitive fertilisation success between competing ejaculates exclusively to the action of chemoattractant (i.e. it allows us to directly link differential chemotactic movement with the fitness outcome of sperm competition). Using eggs from a separate standard female for the fertilisations enables us to make this link by ensuring that within each block, the only source of male x female variation in competitive fertilisation rates is through differential chemoattraction. The standard female eggs, which were the same throughout all cells of the block, would have had no confounding effect on male x female variation. We performed each competition in replicate, i.e. eight competitions per block (Fig. 3.1A), and conducted a total of 11 blocks (i.e.  $n=22$  focal males, 22 focal females, 44 male-female combinations, 88 competitions).

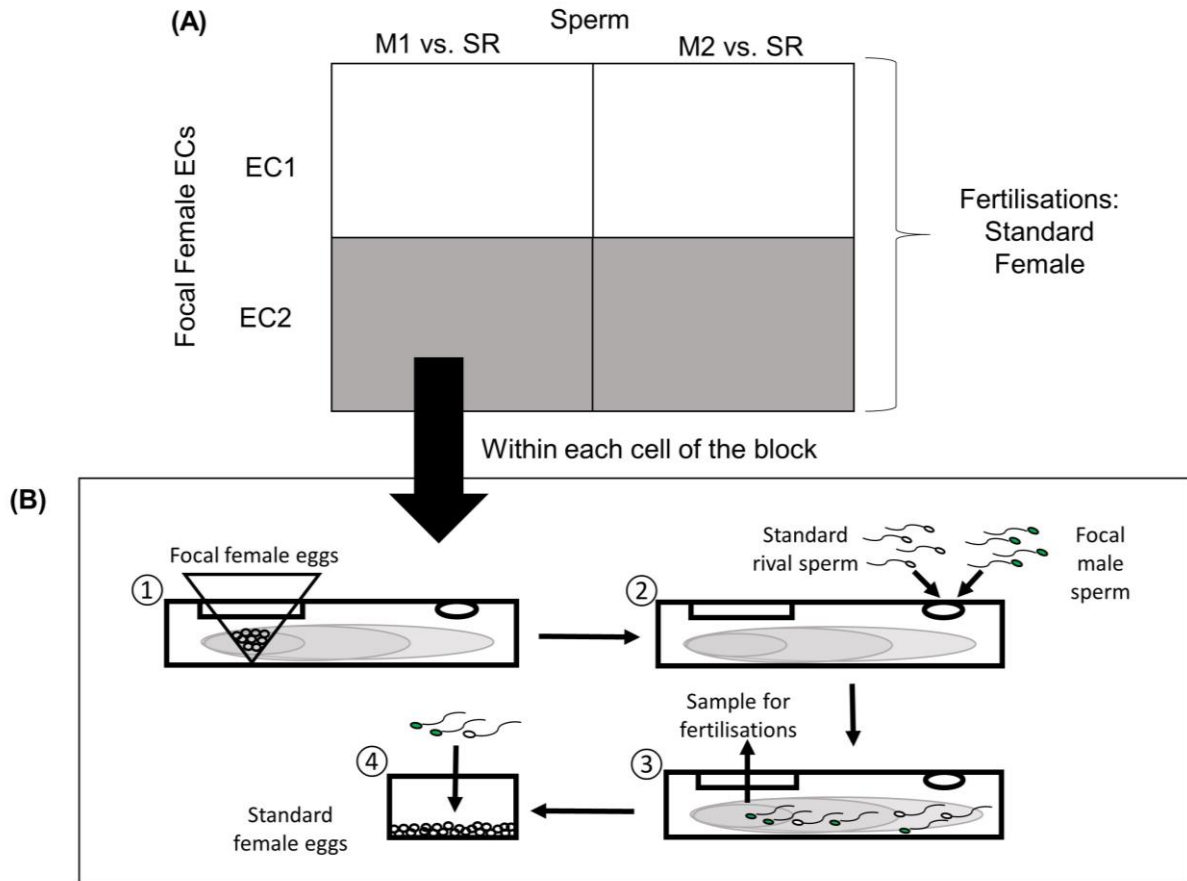
### *Competitive chemotaxis and fertilisation trials*

In the first step of our experimental procedure, we established a chemoattractant gradient in an experimental chemotaxis chamber, then allowed dyed focal (M1 or M2) sperm and undyed rival (SR) sperm to swim in the chamber (Fig. 3.1B; these steps were performed for each cell of Fig. 3.1A). The chambers were made from sterile syringes (Terumo), with the ends of each syringe sawn off and sealed with parafilm (Bemis) to form a 10 mL tube. A  $\sim 2$  cm<sup>2</sup> section was removed at one end of the chamber, and a small hole drilled in the other end. The chambers were fixed to a flat surface and a filter sack made of 30  $\mu$ m filter mesh



was inserted through the square opening. We added 5 mL of FSW to the chamber and 2 mL of egg solution (at  $5 \times 10^4$  cells  $\text{mL}^{-1}$ ) to the filter sack, which retained eggs but allowed chemoattractants to disperse into the chamber. We left the chambers for 1 h to establish a chemoattractant gradient (this time frame has previously been used to establish a chemoattractant gradient in larger chambers and we confirmed in preliminary trials that it was sufficient for our chambers; Evans et al. 2012).

Aliquots of sperm from the focal males and the standard rival were standardised to the same concentration (see below) and prepared for each competitive chemotaxis trial. The focal male's sperm was labelled using MitoTracker Green FM (Molecular Probes), prepared as described in Chapter 2. In our previous study, we showed that dyeing sperm has no effect on sperm behaviour or competitive ability (Chapter 2). Apart from the addition of dye, focal male and standard rival sperm were treated to the same procedure. Briefly, 950  $\mu\text{L}$  aliquots of sperm at  $1 \times 10^6$  cells  $\text{mL}^{-1}$  were prepared from each male, 50  $\mu\text{L}$  of 500 nm dye solution added to focal male aliquots, and 50  $\mu\text{L}$  of FSW added to rival aliquots. All samples (including undyed) were left in the dark (to prevent degradation of dye) for 10 minutes. The filter mesh containing focal female eggs was then removed from each chamber, and 500  $\mu\text{L}$  each of focal male and standard rival sperm solution added to the drilled hole at the opposite end of the chamber (Fig. 3.1B). Sperm were allowed to swim in the gradient for 10 minutes. Preliminary trials confirmed that this assay did not result in any contamination of non-focal sperm by excess dye from focal sperm (see Supplementary Methods).



**Figure 3.1.** The overall design of an experimental block (A), and the steps performed within each cell of the block (B). (A) An example of one cross classified block, in which sperm from each of two focal males (M1 and M2) compete against sperm from a single standard rival (SR) in chemoattractant gradients from each of two focal females (F1 and F2). This generated four combinations per block, which were each replicated ( $n = 11$  blocks, 44 combinations, 88 competitions total). Eggs from a single standard female per block were used to estimate competitive fertilisation success. (B) The multistep competition assay illustrated using a single combination from within a block. (1) Eggs from the focal female were suspended in filter mesh to generate a chemoattractant gradient within the chamber. (2) The mesh and eggs were removed after 1 h, and dyed sperm from the focal male and undyed sperm from the standard rival added to the other end of the chamber. (3) After 10 minutes, a subsample was taken from the centre of the chemoattractant gradient. (4) The subsample was added directly to eggs from the standard female, and competitive fertilisation success of the focal male was measured.

After focal and rival sperm had been in the chemotaxis chamber for 10 minutes, 1 mL samples were taken from the centre of the chemoattractant gradient (see Fig. 3.1B) and added to a separate petri dish containing 1 mL of FSW with eggs from the standard female (diluted to  $1 \times 10^4$  cells  $\text{mL}^{-1}$ ). Prior to the addition of sperm, we rinsed the standard eggs with FSW through 30  $\mu\text{m}$  filter mesh to remove egg chemoattractants. However, even if these standard female eggs subsequently released chemoattractants, their impact (if any) would be to lessen our chance of detecting significant male-by-female effects (by obscuring patterns driven by the chemoattractants of focal females). Therefore, a significant male-by-female interaction in our analysis could only be attributable to the focal chemoattractants, which varied across the focal male samples. Moreover, fertilisation occurs rapidly upon the addition of sperm to the standard eggs (Chapter 2), therefore decreasing the possibility that standard egg chemoattractants could reduce our power to detect effects. Although fertilisation itself was rapid, we waited 10 minutes after the addition of sperm to allow dyed mitochondria to become visible inside fertilised eggs (Chapter 2). We then estimated the fertilisation success of the focal male under a fluorescent microscope by observing haphazard samples of 100 eggs, recording the numbers with and without dyed mitochondria.

Fertilisations from the rival (undyed) male were not scored, as estimating fertilisations from undyed sperm requires eggs to be left until they develop polar bodies, undergo cell division or until they can be assayed for survival. Therefore, the total number of fertilised eggs (dyed plus undyed) were not scored in this procedure. However, this is not required for the interpretation of the effects in our design, as we are not directly comparing the competitive success of focal males to rival males, but rather comparing the competitiveness of different focal males when they compete with a standard rival for standard eggs across different focal chemoattractants. Variation in the number of standard female eggs available for fertilisation overall would only contribute to block-level variation (as all trials within a block used eggs from the same standard female) and therefore would not systematically change the relative share of paternity among focal males within a block. Therefore, the male, female and male x female effects (all nested within block) on competitive fertilisation were not confounded by variation in proportion of standard female eggs available for fertilisation.

### *Nuclear genetic relatedness*

Foot tissue samples from all focal males and focal females (i.e. egg chemoattractant donors) were preserved in 100% ethanol. DNA was extracted using a salt-extraction method as described in Simmons et al. (2006) with the following alterations: tissue samples were incubated at 56°C overnight in the extraction buffer, and extracted DNA was resuspended in 100 µL of sterile water. DNA concentrations were estimated using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and DNA samples were stored at -20°C until required for PCR amplification. Each individual was genotyped at 13 polymorphic microsatellite loci; MGE002, MGE005, MGE008 (Yu and Li 2007), Mgu3 (Presa et al. 2002), Med744 (Lallias et al. 2009), MT282 (Gardestöm et al. 2008), MGES11 (Li et al. 2011), Mg-USC20, Mg-USC22, Mg-USC25, Mg-USC28, Mg-USC42 and Mg-USC43 (Pardo et al. 2011) (primer sequences provided in Table S3.1). Singleplex PCR reactions were run for each sample at each locus with a reaction volume of 5 µL, containing 1 µL MyTaq reaction buffer (Bioline), 0.2 µL primer mix (solution containing 10 nM each of forward and reverse primer, forward primer fluorescently labelled), 0.5 µL bovine serum albumin (Fisher Biotec), 0.1 µL MyTaq DNA Polymerase (Bioline), 2.2 µL sterile water and 1 µL DNA sample (approximately 10 ng). PCRs were performed using an Eppendorf Mastercycler epGradient S, with an initial denaturation step at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 54°C (MGE005 and MGE008) or 60°C (all other loci) for 1 min and 72°C for 1 min, with a final extension step of 72°C for 5 min. The PCR products were analysed on an ABI 3730 96 capillary machine using a Genescan-500 LIZ internal size standard, and genotypes for each locus were scored using GENEMARKER software (SoftGenetics). Peaks identified by GENEMARKER were checked manually and adjusted as necessary to minimise scoring errors.

One locus (MGES11) was monomorphic for our samples, with the number of alleles for the other 12 loci ranging from 3-20. We examined patterns of subpopulation variation and clustering of nuclear genotypes using the software programme STRUCTURE (Pritchard et al. 2000, 2007; Falush et al. 2003; Supplementary Methods). Pairs of loci were tested for genetic linkage using likelihood ratio tests in GENEPOP (Raymond and Rousset 1995; Rousset 2008), with one pair of loci in significant linkage disequilibrium (Med744 and Mg-USC22,  $p < 0.001$ ). We therefore removed one of these loci from the analysis, specifically Med744 as there was also evidence of null alleles at this locus (Table S3.2; null alleles

estimated using MICROCHECKER software; Van Oosterhout et al. 2004). There were excess homozygotes and evidence for null alleles at seven other loci (Table S3.2). However, removing all loci with null alleles can considerably reduce the power to detect variation in genetic relatedness and result in less accurate relatedness estimates than when all loci are included (Supplementary Methods; see also Robinson et al. 2013). We therefore used a maximum likelihood estimator that can account for null alleles (Kalinowski et al. 2006) to calculate genetic relatedness from the remaining 11 loci between each focal male-female pair in each block. These estimates were calculated using the ML-RELATE software package (Kalinowski et al. 2006). We compared these estimates to a range of other relatedness estimators and found consistent patterns of variation in relatedness across different methods, increasing our confidence in the reported measures of nuclear genetic relatedness (see Supplementary Methods). Moreover, to determine whether any markers had a disproportionate effect on measures of relatedness, we examined whether relatedness changed when each marker was removed in turn, and found little variation across different combinations (Table S3.3).

#### *Mitochondrial haplotypes*

We sequenced female-type (F-type) CO1 mtDNA, which is generally considered to have a more reliable phylogenetic signal than male-type mtDNA and has multiple phylogenetic lineages in Australian *M. galloprovincialis* populations (Gérard et al. 2008; Colgan and Middelfart 2011; Dias et al. 2014). Using the DNA extracted as previously described, we amplified F-type CO1 haplotypes using PCR reagents and conditions as described in Dias et al. (2014). Samples were sequenced in both directions by the Australian Genome Research Facility, Perth. Consensus sequences were aligned, analysed and trimmed in Geneious v 6.1.8 (Kearse et al. 2012) using the Geneious alignment feature with default parameters. A preliminary Neighbour-Joining tree was constructed from the 44 individuals to identify the number of unique sequences present ( $n = 14$ ; Table S3.4). We added 105 northern and southern *Mytilus* haplotypes of the COI gene to our unique sequence set, as compiled in Dias et al. (2014). We inferred phylogenetic relationships using MRBAYES V3.1.2 (Huelsenbeck and Ronquist 2001) in Geneious v 6.1.8. We set the parameters and performed the Bayesian analyses as described in Dias et al. (2014), with the modification

that we used a GTR+G substitution model. We determined phylogenetic relationships from 75% majority-rule consensus of post-burn-in trees.

### *Statistical analyses*

Analyses were performed using R version 3.3.2 (R Core Team 2017). We first analysed competitive fertilisation success of focal sperm as a binomial response variable (proportion of eggs successfully fertilised by dyed sperm in competition). We fit a GLMM with logit link function in the 'lme4' package (Bates et al. 2014), using the Laplace approximation of the log-likelihood to estimate model parameters (Raudenbush et al. 2000). Our model included a fixed intercept term and random effects of male (overall variation among sperm of focal males), female (overall variation among focal female chemoattractants), male-by-female interaction (variation among sperm-chemoattractant combinations), and experimental block. There was no overdispersion in our model (residual deviance = 77.15 on 83 degrees of freedom, dispersion parameter = 0.93), and the scaled residuals (calculated using the 'DHARMA' package; Hartig 2017) were uniformly distributed (Kolmogorov-Smirnov test;  $D = 0.053$ ,  $P = 0.967$ ). Focal male competitive fertilisation success ranged from 0% to 44%, i.e. significantly lower than 50% (fixed intercept term of GLMM = -1.79 [95% CIs = -2.11, -1.47], Wald  $Z = -1.78$ ,  $P < 0.001$ ). This was expected given only the subset of sperm that successfully travelled to the centre of the chemoattractant gradient was used for fertilisations. We assessed the significance of random effect terms by removing each from the model in turn and compared the fit of the reduced models against the full model with likelihood ratio tests ( $-2 \times$  difference in log likelihoods compared against  $\chi^2$  distribution with 1 degree of freedom).

Next, we examined whether nuclear genetic relatedness and mitochondrial lineages of focal male and focal (i.e. chemoattractant-producing) female pairs were predictive of competitive fertilisation success. The replicate measures of competitive fertilisation success for each combination of focal sperm and focal chemoattractant were significantly repeatable ( $R = 0.044$  [95% CIs 0.023, 0.069],  $P < 0.001$ ; estimated using GLMM method in the 'rptR' package; Nakagawa and Schielzeth 2010). Therefore, the replicate measures were combined into weighted means (i.e. total fertilised out of total number of eggs across the two replicates). We fit a GLMM with logit link function to competitive fertilisation success, with a

continuous fixed effect of nuclear relatedness and a fixed categorical factor specifying whether the focal male and focal female pair had the same mitochondrial lineage or a different lineage. We also fit random effects of male, female and block. There was no evidence of overdispersion in our model (residual deviance = 11.91 on 37 degrees of freedom, dispersion parameter = 0.32), nor heteroscedasticity of scaled residuals (Kolmogorov-Smirnov test;  $D = 0.079$ ,  $P = 0.944$ ). We used Wald Chi-square tests to assess the significance of the fixed effects.

### **3.4 Results**

#### *Competitive fertilisation success*

There were two sources of significant variation in focal male competitive fertilisation success: (a) the male effect, and (b) the male-by-female interaction (Table 3.1). Although significant interactions often dictate that other effects must be interpreted cautiously, in this case the removal of both the male effect and the male-by-female interaction resulted in a significantly worse fit than removal of the male-by-female interaction alone (likelihood ratio statistic  $G^2 = 68.80$ ,  $p < 0.001$ ). Therefore, the significant male effect suggests that there was variation among males in their average competitive success (i.e. some males were intrinsically 'better' sperm competitors than others). The male-by-female interaction, on the other hand, indicates that there was significant variation in the way chemoattractants of focal females affected the competitive success of different focal males. In other words, the success of each focal male within a block depended on the specific identity of the focal female chemoattractant.

#### *Genetic relationships*

The nuclear data indicated a well-mixed population (Fig. S3.1), despite F-type CO1 mtDNA haplotypes revealing signatures of two historical phylogenetic lineages (consistent with previously identified Northern and Southern Hemisphere lineages; Fig. S3.2; see also Dias et al. 2014). Nuclear genetic relatedness did not differ between focal male-female pairs that had the same mitochondrial lineage and those that had different mitochondrial lineages (two-sample  $t$ -test,  $t_{42} = 0.31$ ,  $p = 0.759$ ). We tested whether overall nuclear genetic relatedness or phylogenetic mtDNA lineages of focal male and focal (i.e. chemoattractant-producing) female pairs predicted patterns of gamete-level sexual selection (i.e. competitive

fertilisation success). We found significant main effects of both nuclear relatedness and mitochondrial lineage (Table 3.2). Specifically, competitive fertilisation success was higher when focal male and focal female nuclear genotypes were less related, but also when focal males and focal females had the same mitochondrial lineage.

**Table 3.1.** Results of log-likelihood ratio tests for random effects on focal male competitive fertilisation success.

Model	Log likelihood	AICc	G <sup>2</sup>	P
Full	-282.94	576.60		
(-Male)	-285.89	580.26	5.90	0.015*
(-Female)	-283.27	575.01	0.66	0.417
(-Male × Female)	-285.41	579.30	4.95	0.026*
(-Block)	-283.84	576.17	1.81	0.178

Full generalised linear mixed-effects model included the proportion of eggs successfully fertilised by the focal male as the response variable (with logit link function), with random effects of focal male ID, focal female ID, male-by-female interaction and experimental block. The fixed intercept of the full model was significantly negative (intercept = -1.79 [95% CIs = -2.11, -1.47], Wald Z = -1.78,  $P < 0.001$ ). Estimated variance components associated with random effects are provided in Table S3.5. Reduced models were fit by excluding each random effect in turn. Akaike information criteria with correction for finite sample sizes (AICc) are provided for full and reduced models. The likelihood ratio statistic ( $G^2$ ) for each random effect was calculated as  $-2 \times$  difference in log-likelihoods between the relevant reduced model and the full model. Probability ( $P$ ) statistics were estimated by comparing  $G^2$  to a  $\chi^2$  distribution with one degree of freedom.



**Table 3.2.** Effects of nuclear genetic relatedness and phylogenetic mitochondrial lineage on competitive fertilisation success.

Fixed effect	Estimate	$\chi^2$	<i>P</i>
Nuclear relatedness	-0.35 [-1.32, -0.02]	3.92	0.047
Mitochondrial lineage	0.35 [0.22, 0.65]	15.52	<0.001

Effects estimated from generalised linear mixed-effects models of the proportion of eggs successfully fertilised by the focal male (with logit link function), with fixed effects of nuclear relatedness and mitochondrial lineage and random effects of focal male ID, focal female ID, and experimental block. The final model did not include the interaction term of the fixed effects, as the interaction was non-significant in the full model (Wald  $\chi^2 = 0.93$ ,  $P = 0.335$ ) and its inclusion reduced model fit (see Table S3.6; although significance of the main effects did not change with inclusion of the interaction). The fixed intercept of the model was significantly negative (intercept = -1.58 [95% CIs = -1.95, -1.22], Wald  $Z = -9.08$ ,  $P < 0.001$ ). Nuclear relatedness of focal male and focal female pairs was estimated from microsatellite loci using maximum likelihood (higher values = more closely related). Mitochondrial lineage (Northern or Southern Hemisphere) was assigned based on female-type CO1 sequences, with focal male and focal female pairs scored as belonging to different or same lineage (estimate represents the mean change in fertilisation success on the latent scale from different to same lineage). Hypothesis tests of main effects were conducted using Wald  $\chi^2$  tests (d.f. = 1 for each effect).

### 3.5 Discussion

Our results reveal that differential attraction of sperm up a chemical gradient can act as a mechanism of gamete-level mate choice. To our knowledge, this is the first direct evidence that egg chemoattractants influence intraspecific sperm competition, supporting the previously documented differential effects of egg chemoattractants on sperm swimming direction (Evans et al. 2012), sperm motility (Oliver and Evans 2014) and sperm physiology (Kekäläinen and Evans 2016). We show that the effect of chemoattractants on competitive fertilisation success depends upon the particular combination of focal male and focal female, specifically favouring certain genetic combinations over others. Previous work on this system has shown that the strength of sperm chemotactic responses for any given male-female pairing is positively correlated with offspring survival (Oliver and Evans 2014). These previous findings, together with the present results, suggest that egg chemoattractants allow females to promote fertilisation by more compatible males when

multiple ejaculates compete. This provides rare insight into the mechanisms used by females to gain control over the outcome of sperm competition.

Our results complement and extend recent evidence that female reproductive fluids more broadly can have important roles in gamete-level sexual selection. In particular, there has been considerable interest in the ovarian fluid (OF) produced by various female fishes. In externally-fertilising salmonids, for example, OF released with eggs can differentially mediate the swimming speed of conspecific sperm depending on the particular male-female pairing (Urbach et al. 2005; Rosengrave et al. 2008; Butts et al. 2012). Although OF has yet to be implicated in intraspecific gamete-level mate choice in salmonids (Evans et al. 2013), it has been shown to promote fertilisation by conspecific sperm when in competition with those of sister species (Yeates et al. 2013). Intriguingly, however, there is evidence from an internally-fertilising poeciliid fish that OF within the female's reproductive tract can selectively bias fertilisation in favour of sperm from unrelated males over related males (Gasparini and Pilastro 2011). Recent work on an externally-fertilising wrasse has also shown that OF can bias competitive fertilisation success towards dominant 'nest' males (i.e. directional cryptic female choice; Alonzo et al. 2016). Our findings for mussels complement these prior studies by showing that egg chemoattractants similarly play an important role in mediating intraspecific sperm competition, thus exposing a previously unforeseen mechanism of sexual selection that may occur more broadly in other taxa. We suggest that further investigation into the effects of female reproductive fluids, including egg chemoattractants, across a broader range of taxa will provide fruitful mechanistic insights into gamete-level mate choice.

We also found that the competitive fertilisation biases induced by egg chemoattractants reflect complex genetic relationships between the focal males and focal (i.e. chemoattractant-producing) females. These results may shed some light on patterns of genetic compatibility that underlie competitive fertilisation biases, given previous findings that differential chemotaxis is correlated with offspring fitness of male-female pairs (Oliver and Evans 2014). Competitive fertilisation success was higher for focal males that had a lower overall genetic relatedness to focal females (based on neutral nuclear markers), which complements recent evidence in other taxa that preferences for genetically dissimilar males

may drive compatibility-based cryptic female choice (Gasparini and Pilastro 2011; Firman and Simmons 2015). Although we did not directly examine the extent of inbreeding in our population, homozygote excesses consistent with inbreeding are not uncommon in populations of broadcast spawners (Huang et al. 2000; Addison and Hart 2005; Kenchington et al. 2006), possibly due to the unpredictable patterns of spawning and recruitment in these systems (Hedgecock and Pudovkin 2011). Therefore, gamete-level mechanisms of maximising offspring heterozygosity may be important for individual reproductive fitness.

In contrast to the patterns of overall genetic relatedness, we also found a competitive fertilisation bias toward males that had the same phylogenetic mitochondrial lineage as the female. Preferences based on phylogenetic lineage are not unexpected in Australian *M. galloprovincialis* populations, as Northern and Southern Hemisphere lineages had diverged in allopatry from the Pleistocene before the more recent introduction of Northern individuals (Hilbish et al. 2000; Gérard et al. 2008). Nevertheless, it appears that such preferences have not maintained reproductive isolation between lineages, with the admixture of nuclear genotypes in our population supporting previous findings for Australian populations (Westfall and Gardner 2013). Possibly, this could be due to lineage-based patterns being offset by the preferences for less related nuclear genotypes. However, the precise fitness benefits of the mitochondrial lineage-based biases deserve further investigation. For example, one possibility is that fertilisation biases reflect cyto-nuclear compatibilities brought about by the presence of divergent mitochondrial lineages; it would therefore be interesting to examine how preferences relate to nuclear genes involved in mitochondrial function. Moreover, we sequenced the female-type mtDNA common to somatic tissues of both males and females, but the occurrence and transmission of male-type mitochondria in sperm may further complicate patterns. Therefore, the precise genetic interactions between males and females that underlie chemoattractant-driven fertilisation biases in these systems remain to be fully resolved.

In order to provide further mechanistic insights into gamete-level mate choice in this system we need to identify the chemical profiles of egg chemoattractants and determine how variation in these profiles correspond to patterns of differential sperm attraction. Chemoattractant molecules have not yet been identified in *M. galloprovincialis*, but several

types of egg-derived chemicals have been described in other broadcast spawners (reviewed in Evans and Sherman 2013). For example, in echinoderms, peptides released from eggs bind to guanylyl cyclase receptors on the sperm surface, triggering a signalling pathway that results in influxes of extracellular calcium ions and a corresponding flagellar beat pattern (Kaupp et al. 2006; Alvarez et al. 2014). However, to our knowledge there has been no examination of intraspecific variation in such signalling pathways in any species. Recent evidence suggests that sperm-activating peptides are evolutionarily conserved and vary little within genera (Jagadeeshan et al. 2015). Therefore, it may be unlikely that a single molecule type (such as a particular peptide) is responsible for intraspecific variation in sperm chemoattraction. Instead, it is possible that eggs release a variety of molecules that affect such signalling pathways. Our finding that the interacting effects of parental genotypes drive chemoattractant preferences suggests that these chemical signals are likely to be complex. Clearly there is a need to characterise intra-specific variation in egg chemoattractant chemical profiles in order to address these questions.

In conclusion, we provide the first direct evidence that egg chemoattractants moderate sperm competition and complement these findings with genetic data that may explain the previously documented offspring fitness benefits associated with differential sperm chemotaxis (Oliver and Evans 2014). Given our focus on a species exhibiting the ancestral mating strategy of broadcast spawning, and the fact that egg chemoattractants are found throughout a diverse range of taxa (Miller 1985; Eisenbach 1999; Teves et al. 2009), we anticipate that such mechanisms of gamete-level mate choice may be prevalent in other species. However, until now the putative role of sperm chemotaxis in mediating intraspecific sperm competition has been largely untested. This is likely due in part to the empirical difficulty of linking the effect of putative mechanisms of gamete-level mate choice directly to variation in competitive fertilisation success. We demonstrate that powerful and tightly controlled experimental designs can provide detailed insights into the intricacies of gamete-level sexual selection.

### **3.6 Acknowledgements**

We thank Jukka Kekäläinen for assistance with mussel collections, Stephen Robinson and Catherine Seed for helping design and create the chemotaxis chambers, Yvette Hitchen for

assistance with microsatellite and mtDNA analyses, and Alan Lymbery and two anonymous reviewers for helpful comments on the manuscript. Funding was provided by the UWA School of Animal Biology (RAL) and the Australian Research Council (JPE; Grant Number DP150103266). RAL was supported by the Hackett Postgraduate Research Scholarship and the Bruce and Betty Green Postgraduate Research Top-Up Scholarship.

### 3.7 Supplementary Materials

#### Supplementary Methods

##### *Sperm dye contamination trials*

We conducted preliminary trials to determine whether adding dyed focal sperm solution and undyed rival sperm solution to the chemotaxis chamber would result in any contamination of rival sperm cells with dye. In these trials, we prepared dyed and undyed sperm samples following the same procedure as described in the main text. We then centrifuged the dyed sperm sample and collected the supernatant; i.e. the part of the dyed sample that would contain any excess dye solution but not the sperm cells themselves. This supernatant was then added to 7 mL of filtered seawater (i.e. replicating the volume of the chemotaxis chambers) along with the prepared undyed sperm sample. After 10 minutes (i.e. the length of time sperm were allowed to swim in the chemotaxis chamber in the experiment), we took subsamples from this solution and checked them under a Zeiss Axio Imager A1 fluorescent microscope. In haphazard counts of 100 sperm cells identified under normal light microscope, we did not find any contaminated by dye (n = 5 trials).

##### *Testing for subpopulation structure of nuclear genotypes*

We tested for subpopulation structure by analysing the microsatellite data with the software programme STRUCTURE (Pritchard et al. 2000, 2007; Falush et al. 2003). This programme uses Bayesian clustering analysis to assign individuals to K genetically distinct clusters. We did not use prior information about the location of samples (as all individuals were sampled from the same location). Our model parameters assumed admixture and correlated allele frequencies. We conducted 10 independent runs each for values of K ranging from 1-5, each with a burn-in of 10,000 followed by 100,000 Markov chain Monte Carlo (MCMC) iterations. We determined the most likely number of clusters by comparing the log probability of models with different values of K and the rate of change in log probability between successive values of K ( $\Delta K$ ) (Evanno et al. 2005).

##### *Comparing performance of genetic relatedness measures*

To check that our observed patterns of genetic relatedness were not an artefact of the relatedness estimator that we report in the main text (a maximum likelihood estimator that

can account for null alleles, here denoted as ML-r), we compared a range of other relatedness measures for our loci. We first compared the performance of five different moment estimates of relatedness using the 'compareestimators' function of the 'related' R package (Pew et al. 2015). Briefly, the program uses observed genotype data to simulate genotypes from individuals of set levels of relatedness (full-sib, half-sib, parent-offspring and unrelated), and then compares the pairwise estimates of relatedness for these simulated individuals to theoretical expectations (0.5 for full-sib and parent-offspring, 0.25 for half-sib, 0 for unrelated). The correlation between the simulated relatedness estimates and the theoretical expectations can be used to assess the performance of each particular estimator for the observed loci. We found that all five moment estimators correlated strongly with theoretical expectations ( $r$  ranged from 0.70 - 0.76 for the five estimators), with the estimators of Lynch and Ritland (1999) (here denoted LR;  $r = 0.76$ ) and Queller and Goodnight (1989) (QG,  $r = 0.71$ ) best matching theoretical expectations. Note that if we included only observed genotypes at loci that did not show evidence of null alleles (see Supplementary Table 2) in the analysis, then the relatedness estimators performed more poorly against theoretical expectations (correlation  $r$  between estimators and theoretical expectations ranged from 0.49 - 0.52). This validates our expectation that removing these loci would reduce the power to detect variation in genetic relatedness and decrease the accuracy of estimators (Robinson et al. 2013).

The 'related' program can also determine the performance of a further two maximum likelihood methods of calculating relatedness, a dyadic likelihood method (Milligan 2003) and a triadic likelihood method (Wang 2007). However, these are computationally intensive to simulate and can only be compared to other estimators using manual code. We therefore compared only the best two moment estimators (as described above) to the two maximum likelihood estimators using manual code provided by Frasier (Frasier 2015). We found that the maximum likelihood estimators performed similarly to the moment estimators ( $r$  ranged from 0.70 - 0.72, note these are slightly different from the previous correlation coefficients when using the moment estimators only due to the random nature of simulations). We therefore used the best maximum likelihood estimator (triadic estimator, Tri) and the best two moment estimators (LR and QG) in further comparisons.

We determined whether the pairwise relatedness estimates provided by LR, QG and Tri were similar to pairwise relatedness calculated by ML-r (as reported in the main text). We used mantel tests to compare pairwise genetic relatedness matrices between the methods, and found that all three alternative methods from the 'related' package correlated strongly with the ML-r estimates (LR:  $r = 0.78$ ,  $p < 0.001$ ; QG:  $r = 0.71$ ,  $p < 0.001$ ; Tri:  $r = 0.85$ ,  $p < 0.001$ ). The consistent patterns across various relatedness estimators, together with the concordance with theoretical expectations in simulations, increases our confidence in the results we obtained using the ML-r estimator that accounts for null alleles (as presented in the main text).



## Supplementary Tables

**Table S3.1.** Primer sequences, size range (Bp, base pairs) expected from literature (observed size range in parentheses) and references for the 13 microsatellite loci for *M. galloprovincialis* used in this study.

Locus	Primer sequence	Bp range	Reference
MGE002	F: GGTAGTTGGAGTGGTTGGT R: AATGGTCGGTAGATGAAAAT	272-280 (269-290)	(Yu and Li 2007)
MGE005	F: CGTTGCCATCGTTTATTTT R: GTTGTAAGTCGTGTTGGTTCA	220-244 (226-247)	(Yu and Li 2007)
MGE008	F: TGCTAAAAGTAATAAGACAGAT R: GAGACCTCCAATAAATAAAA	268-286 (272-280)	(Yu and Li 2007)
Mgu3	F: AACTAAAAACTTCATCTAATCCC R: AAGCAATCCAAAGTGAGAGG	143-151 (134-148)	(Presa et al. 2002)
Med744	F: TTTTTCATCGTGTTTTGGTTG R: CGCCATGGAATAGCCAATAG	220-296 (188-231)	(Lallias et al. 2009)
MT282	F: TGCCACATTGTTTTCAAGGA R: TTCACGACAGCGACTATGAAA	336-354 (332-350)	(Gardestöm et al. 2008)
MGES11	F: CATCCCCGTATGGACATCAAG R: ATCTGACACTGTGCAAATTGAGATC	191-254 (180-180)	(Li et al. 2011)
Mg-USC20	F: TACAGAAACGCCATGTCAGC R: TGAGGTTCAAAGAACGGAAGA	284-308 (284-318)	(Pardo et al. 2011)
Mg-USC22	F: CCACAGGCACAACAAGTGTC R: GCCACGTCTATAATGGCAGAA	284-308 (380-384)	(Pardo et al. 2011)
Mg-USC25	F: TCAGGTATCGTGACGGAAAT R: GCAATAGATGCGCCTTCTTC	272-308 (251-276)	(Pardo et al. 2011)
Mg-USC28	F: CCACTGGACGTAAAGCAACC R: CAAGCTCAATAGCTGGCTGA	169-181 (161-169)	(Pardo et al. 2011)
Mg-USC42	F: CTACCGGGCCTCATTATCA R: GCATCGTATTACCGGAGCAT	138-146 (130-136)	(Pardo et al. 2011)
Mg-USC43	F: TCCAAGATGGTTTAGCATTGG R: TGGTGTATCCCTCCATGACT	207-217 (199-212)	(Pardo et al. 2011)

Polymerase chain reaction conditions are reported in the main text.

**Table S3.2.** Tests for null alleles at 13 microsatellite loci for *Mytilus galloprovincialis*, performed with Bonferroni correction for multiple tests using MICROCHECKER (Van Oosterhout et al. 2004).

Locus	Observed homozygotes	Expected homozygotes	Null alleles present	Estimated null allele frequency
MGE002	37	32.11	Yes	0.18
MGE005	23	8.96	Yes	0.21
MGE008	31	17.77	Yes	0.25
Mgu3	24	21.24	No	NA
Med744	18	8.80	Yes	0.15
MT282	27	9.33	Yes	0.26
MGES11	44	44	NA	NA
Mg-USC20	20	19.77	No	NA
Mg-USC22	21	21.25	No	NA
Mg-USC25	30	23.70	Yes	0.20
Mg-USC28	35	33.10	Yes	0.14
Mg-USC42	18	18.33	No	NA
Mg-USC43	21	12.41	Yes	0.15

Numbers of observed and expected homozygotes are reported, as well as estimated null allele frequencies if present. Note that locus MGES11 was monomorphic and provided no information for relatedness analyses, while locus Med744 was not included in relatedness analyses as it was in linkage disequilibrium with locus Mg-USC22 (see main text).

**Table S3.3.** Mean and standard error of maximum likelihood genetic relatedness of focal male – focal female pairs, estimated from all 11 microsatellite markers used in the final analysis and combinations with each marker removed in turn.

Marker combination	Mean relatedness	SE relatedness
All	0.125	0.024
MGE002 removed	0.136	0.025
MGE005 removed	0.132	0.027
MGE008 removed	0.119	0.024
Mgu3 removed	0.127	0.028
MT282 removed	0.127	0.024
Mg-USC20 removed	0.120	0.023
Mg-USC22 removed	0.132	0.027
Mg-USC25 removed	0.138	0.026
Mg-USC28 removed	0.121	0.024
Mg-USC42 removed	0.134	0.026
Mg-USC43 removed	0.123	0.025

**Table S3.4.** F-type CO1 haplotypes of *Mytilus galloprovincialis* recorded in our study.

Haplotype	Number of individuals	Previously recorded	Lineage	GenBank accession number
H1	2	Haplo7	Northern	KF705219
H2	8	Haplo8	Northern	KF705220
H3	3	Haplo4	Northern	KF705216
H4	15	Haplo1	Northern	KF705213
H5	1	Haplo9	Northern	KF705221
H6	2	Haplo5	Southern	KF705217
H7	1	Haplo6	Southern	KF705218
H8	2	NA	Southern	MF462182
H9	5	Haplo3	Southern	KF705215
H10	1	NA	Southern	MF462183
H11	1	NA	Southern	MF462184
H12	1	NA	Southern	MF462185
H13	1	Haplo14	Southern	KF705226
H14	1	Haplo12	Southern	KF705224

Number of individuals per haplotype are reported, along with previously recorded haplotype IDs if available (as per Dias et al. (2014), haplotype lineage and GenBank accession numbers (for both new and previously recorded haplotypes).

**Table S3.5.** Link-scale approximation of variance components associated with random effects in the full generalised linear mixed model of competitive fertilisation success.

Random effect	Variance component estimate
Male	0.144
Female	0.028
Male x Female	0.051
Block	0.131
Residual*	7.525

Full generalised linear mixed-effects model included the proportion of eggs successfully fertilised by the focal male as the response variable (with logit link function), with random effects of focal male ID, focal female ID, male-by-female interaction and experimental block. The significance of random effects was tested with log-likelihood ratio tests (see main text).

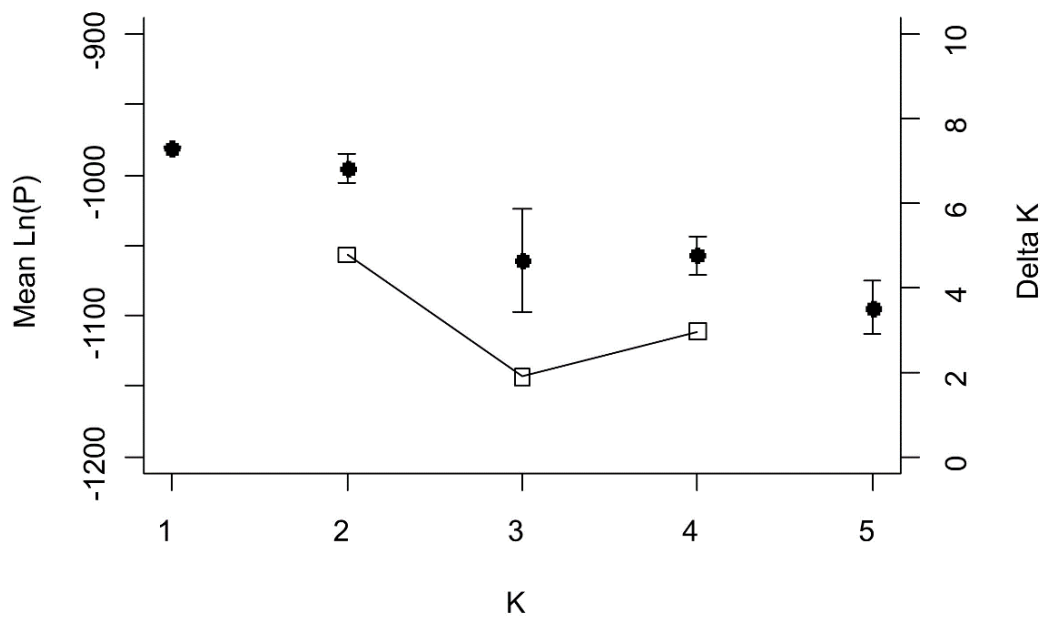
\*The link-scale approximation of the residual variance was estimated using the 'rptR' package (see description of method in Nakagawa and Schielzeth 2010; Schielzeth and Nakagawa 2013).

**Table S3.6.** Comparison of models with different combinations of the fixed effects nuclear genetic relatedness, mitochondrial lineage and their interaction on competitive fertilisation success.

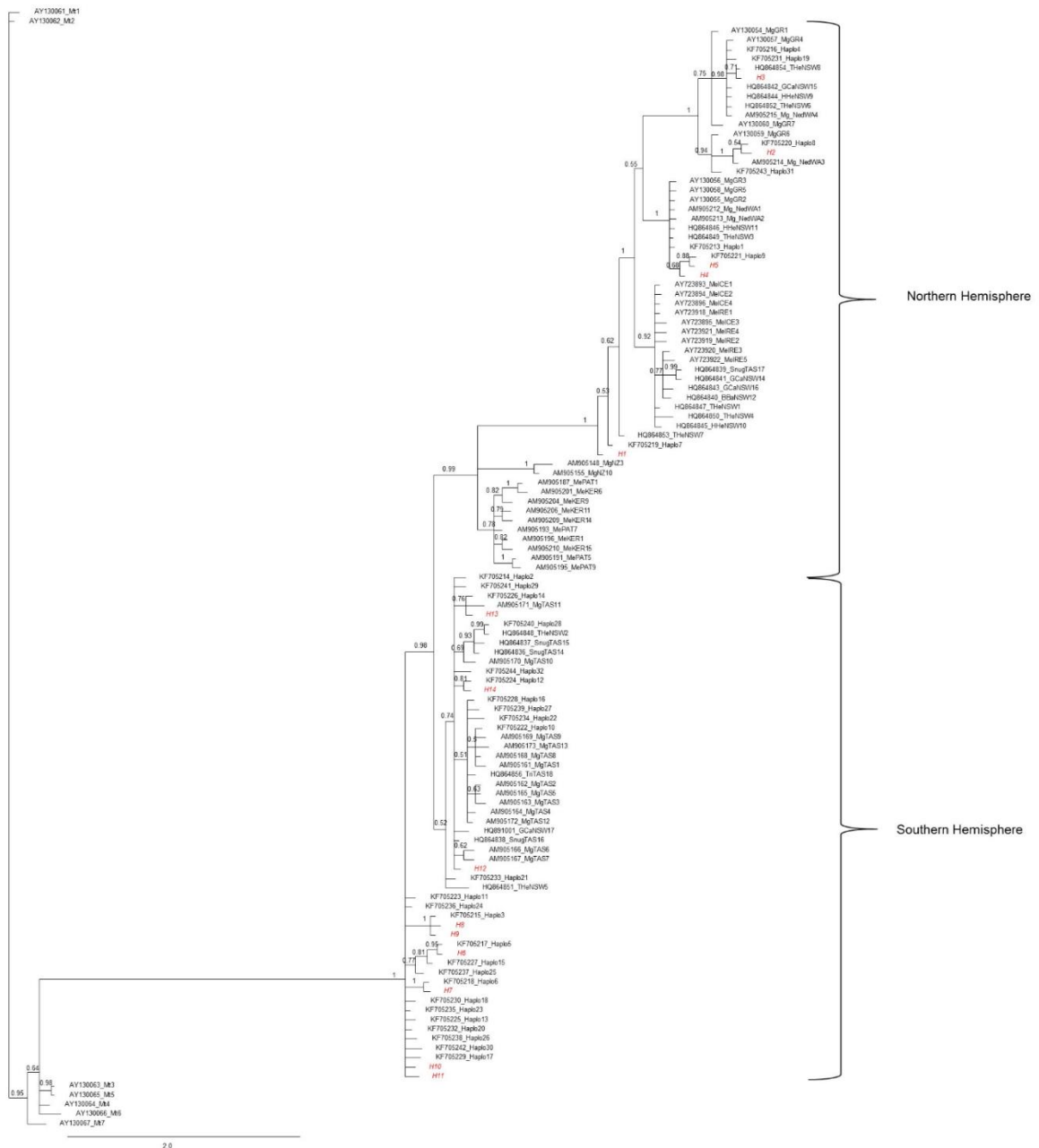
Fixed effects	d.f.	$\Delta AICc$	Weight
Relatedness, Lineage, Relatedness x Lineage interaction	37	1.74	0.203
Relatedness, Lineage	38	-	0.535
Relatedness	39	3.56	0.001
Lineage	39	1.43	0.261

Residual degrees of freedom, difference in corrected Akaike Information Criterion (AICc) between each model AIC and the minimum AIC (model including nuclear relatedness and mitochondrial lineage), and Akaike weights are presented for each model. The best-supported model included nuclear relatedness and mitochondrial lineage, but no interaction. Note that both nuclear relatedness and mitochondrial lineage had a significant effect on competitive fertilisation success (Wald  $\chi^2$  tests; see main text), regardless of whether the interaction term was included.

## Supplementary Figures



**Figure S3.1.** Bayesian modelling of subpopulation structure in microsatellite data, comparing the probability of models with different numbers of clusters ( $K = 1-5$ ). Points represent estimated mean ( $\pm$  standard deviation) log probability (solid circles) of models for each specified  $K$ , and rate of change in log probability between successive values of  $K$  (Delta  $K$ , open squares). The best supported number of clusters was  $K = 1$ ; log-probability of models decreased as the number of hypothesised genetic clusters ( $K$ ) increased. Moreover, in models with  $K > 1$  most individuals were admixed, which is expected in the absence of real subpopulation structure (Pritchard et al. 2007).



**Figure S3.2.** Bayesian phylogenetic tree for *Mytilus* spp. female-type CO1 mitochondrial DNA haplotypes, rooted in *M. trossulus* haplotypes. The 14 unique haplotypes from our study (H1-H14) are shown in red and italics. Other haplotypes (105) were added from those compiled in Dias et al. (Dias et al. 2014); accession numbers for these sequences are provided in the names. Northern and Southern Hemispherelineages are identified on the right hand side.

## CHAPTER FOUR

### **Egg chemoattractants induce differential gene expression in sperm**

*In preparation for submission to a peer-reviewed journal.*

*This chapter is presented as a manuscript for publication, with formatting and referencing consistent with previous and subsequent chapters.*





#### 4.1 Abstract

The fate of sperm cells in their quest to fertilise a female's eggs is typically determined by a complex array of environmental and ecological factors, including the presence of sperm from rival males and female-induced mechanisms that bias fertilisation in favour of particular sperm. A striking example of female-induced sperm selection is provided by the recently discovered phenomenon of differential sperm chemotaxis, where egg-derived chemicals, known as egg chemoattractants (ECs), selectively bias fertilisation towards sperm from particular males. Recent studies in the broadcast spawning mussel *Mytilus galloprovincialis* have demonstrated considerable intraspecific variation in the effects of ECs on sperm swimming behaviour and physiology. Here, we explore the molecular mechanisms of sperm chemotactic responses in *M. galloprovincialis* using a genome-wide comparative analysis of transcription. We assembled a reference transcriptome of 108,138 protein-coding transcripts from gonad tissue and spawned gametes, and found a distinct set of genes enriched in sperm compared to gonads (14,666 genes upregulated in sperm). We then compared gene expression in sperm in the presence and absence of ECs (excluding any transcripts present in ECs themselves), and found that 57 genes were differentially expressed in sperm in response to ECs (34 downregulated and 23 upregulated). A number of these genes matched functional protein categories likely to be involved in known responses to chemoattractants, including signal reception, sperm motility, capacitation and the acrosome reaction. We also detected several protein categories that suggest novel sperm responses to ECs, including the promotion of sperm longevity and the dissolution of semen clots. Our results provide the first explicit evidence of differential gene expression in mature, ejaculated sperm, and suggest that complex molecular mechanisms underlie the phenotypic responses of sperm to environmental cues.

## 4.2 Introduction

The function of sperm cells appears deceptively simple: to deliver their haploid genome to an egg. As such, sperm are often thought of as DNA-delivery machines, with fixed phenotypes determined by males during spermatogenesis. However, between ejaculation and fertilisation, sperm cells are affected by a complex array of physical and biotic factors. There is growing evidence that the post-ejaculatory environment experienced by sperm can result in striking changes to ejaculate phenotypes. For example, studies have revealed that interactions among competing rival sperm (Lüpold et al. 2012; Locatello et al. 2013) or among sperm and females/eggs (Urbach et al. 2005; Rosengrave et al. 2008; Simmons et al. 2009; Gasparini and Pilastro 2011; Kekäläinen and Evans 2016) can differentially modify ejaculate traits such as sperm velocity, motility and capacitation. Such post-ejaculatory effects on sperm phenotypes have potentially important fitness implications for both males and females (and possibly for resulting offspring; e.g. Ritchie and Marshall 2013). However, the specific mechanisms that underlie these interactions are poorly resolved (Pitnick et al. 2009), and we currently lack a detailed understanding of the regulatory factors that control sperm function and behaviour.

Phenotypic plasticity (modification of phenotypes in response to environmental factors) is typically mediated by changes in gene regulation and expression (Schlichting and Smith 2002; Aubin-Horth and Renn 2009; Schlichting and Wund 2014). However, mature sperm cells are generally assumed to be transcriptionally and translationally silent. This is based largely on observations of sperm development in mammals and *Drosophila*, where compaction of sperm chromatin in late spermatogenesis (through replacement of histones with protamines) is assumed to make DNA inaccessible for transcription (Hecht 1998). Nevertheless, there is evidence from these systems that some histones are retained at sequence-specific sites in sperm genomes (Gatewood et al. 1987), and that translation of proteins can occur in mature sperm prior to fertilisation (Gur and Breitbart 2006; Fischer et al. 2012). Even more intriguing are the results of recent studies in several taxa revealing that sperm contain stable populations of coding and non-coding RNAs (Dadoune 2009; Hosken and Hodgson 2014). Although it is commonly assumed that these RNA populations are loaded into sperm by the male, there is growing evidence of post-meiotic transcription in developing sperm themselves (Joseph and Kirkpatrick 2004; Vibrationovski et al. 2010). In

addition, several studies have linked sperm RNA profiles to fertility and fertilisation, indicating that they contribute to fitness and are not merely remnants of spermatogenesis (Dadoune 2009; Jodar et al. 2013). This raises the possibility that regulated gene expression could confer plasticity in sperm behaviour, which might (a) benefit males by allowing sperm to maximise performance across a range of environments, and/or (b) benefit females by allowing them to alter sperm behaviour based on mate preference.

A striking case of female-mediated ejaculate plasticity is sperm chemotaxis, where sperm modify their swimming speed and direction in response to egg-derived chemoattractants. Sperm chemotaxis is widespread throughout internally and externally fertilising taxa, and has been primarily viewed as a means of increasing the target size of eggs (Jantzen et al. 2001; Riffell et al. 2004) or maintaining reproductive isolation through species-specificity of chemoattractants (Miller et al. 1994; Miller 1997). However, recent studies in the broadcast-spawning mussel *Mytilus galloprovincialis* have provided evidence for differential intraspecific effects of egg chemoattractants (ECs) on ejaculates, a phenomenon coined *differential sperm chemotaxis* (Evans and Sherman 2013). Specifically, this work has shown that the effect of ECs on sperm swimming behaviour (velocity, straightness and direction; Evans et al. 2012; Oliver and Evans 2014) and sperm physiology (extent of acrosome reaction and capacitation; Kekäläinen and Evans 2016) depends upon the particular identities of both the male ejaculate donor and the female chemoattractant donor. These male-by-female effects on sperm phenotype correlate with differences in fertilisation success and offspring survival among in vitro crosses (Oliver and Evans 2014), and allow females to bias fertilisation success toward genetically compatible males when multiple ejaculates compete (see Chapter 3). These effects suggest a high degree of plasticity in the responses of sperm phenotypes to different chemotactic signals.

The precise molecular mechanisms underlying individual-specific plasticity in sperm chemotaxis have yet to be established. Studies of sea urchin sperm have identified elements of a signalling pathway induced by the binding of chemoattractant peptides to sperm surface receptors (Kaupp et al. 2006; Alvarez et al. 2014), although intraspecific variation in such signalling pathways has not been described. Simple lock-and-key mechanisms of chemoattractant molecules and sperm surface receptors are unlikely to account for the

wide range of phenotypic responses observed in sperm as these molecules typically show little variation within species (or even within genera; Jagadeeshan et al. 2015). However, tantalising recent evidence suggests that sperm RNA profiles may play an important role in sperm chemotaxis. For example, molecular functions of sperm-enriched transcripts that are involved in sperm chemotaxis, capacitation, acrosome reaction and sperm-egg interactions have been identified (Das et al. 2013). Given that the plastic responses of sperm to different ECs involve these same phenotypic processes (Oliver and Evans 2014; Kekäläinen and Evans 2016), this raises the possibility that gene regulation might play a role in sperm chemotaxis.

Here, we use a genome-wide comparative analysis of transcription to investigate whether gene expression of ejaculates changes in response to ECs in *M. galloprovincialis*. First, we sequence and compare the transcriptome (the full set of expressed transcripts in a sample) of gonad tissue to that of mature, ejaculated sperm to identify sperm-enriched or sperm-specific transcripts in this species. Next, we compare the transcriptomes of active sperm in the presence (treatment) and absence (control) of ECs, to determine whether there are any changes in gene expression when sperm undergo chemotactic responses. We also sequence and control for any RNA present in the chemoattractant samples derived from eggs, ensuring that any remaining differences between RNA profiles of control and treatment sperm samples are attributable solely to differential gene expression by sperm. Finally, we search for biological functions of differentially expressed transcripts and interpret them in the context of the phenotypic processes involved in sperm chemotaxis. Our study provides the first test for differential post-ejaculation gene expression in sperm.

### **4.3 Methods**

#### *Study species and spawning*

*Mytilus galloprovincialis* is a gonochoristic, broadcast spawning mussel that forms large aggregations in intertidal habitats. Within Australian populations, which occur across the southern coastline of the country, mitochondrial DNA lineages from the Northern and Southern Hemispheres have been found (Westfall and Gardner 2010; Dias et al. 2014), though nuclear markers show that these lineages are well-mixed and lack reproductive barriers between them (Westfall and Gardner 2013; see also Chapter 3).

For this study, live mussels were collected from Woodman Point, Western Australia (32°14' 03.6"S, 115°76' 25"E) during the 2015 spawning season (June-September). Individuals were housed in aquaria of recirculating seawater until required for experiments (within one week of collection). As many other marine organisms use mussel shells as substrates, which could lead to contamination of RNA samples from non-target species, we cleaned attached organisms from the shells of mussels immediately prior to experiments. To induce spawning, we transferred mussels to a water bath containing filtered seawater (FSW) heated to 28°C (Oliver and Evans 2014; Kekäläinen and Evans 2016; see also Chapter 2). Once an individual began spawning, it was immediately washed to remove any contaminating gametes from the spawning tank, and placed in an individual sterile 250 mL jar. We covered each mussel with FSW that had been through a second filtration step (using a Millex sterile 0.22 µm syringe filter). Within 30 minutes of the onset of spawning, mussels were removed from the jars and the gamete concentrations were calculated, using an improved Neubauer haemocytometer for sperm (in subsamples fixed with 1% buffered formalin) and counts of cells in a known volume for eggs. We then adjusted gamete concentrations to those required for experimental trials (see below).

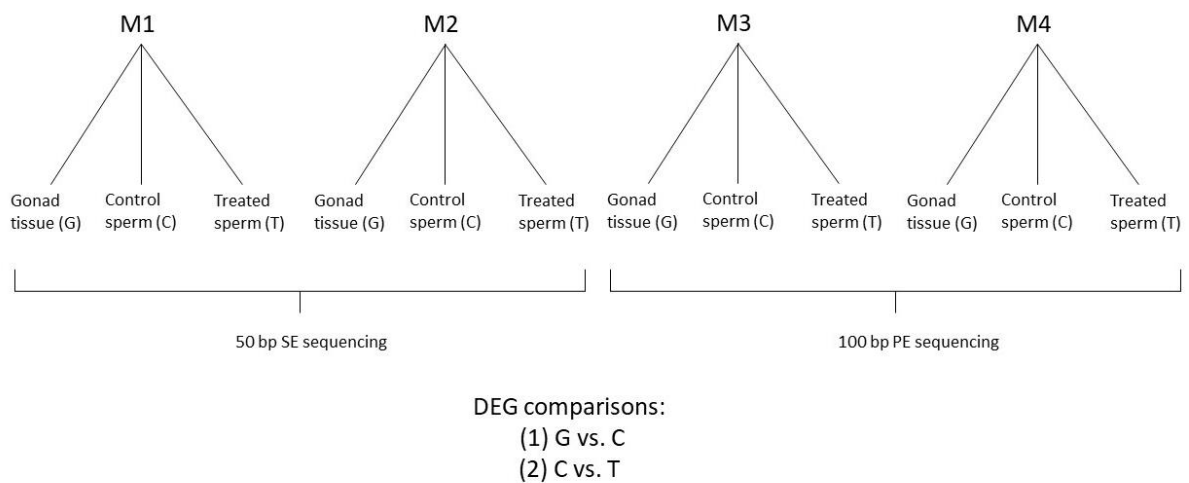
#### *Experimental design and sample collection*

We collected three different sample types from each of four individual males (i.e.  $n = 12$  samples total, four biological replicates per sample type): (1) gonad tissue (G); (2) control sperm (C), i.e. sperm that had only been in contact with twice-filtered seawater; and (3) treated sperm (T), i.e. sperm that had been in contact with 'egg water' (twice-filtered seawater and ECs) (Fig. 4.1). Gonad tissue was extracted from each male after they were removed from their individual spawning jars (see above), frozen using liquid nitrogen, and stored at -80 °C until required for RNA extractions. From the spawning jars, we collected two subsamples of sperm from each male. Sperm cells contain very small amounts of RNA, and high numbers of cells are typically required to extract viable yields for downstream analysis (Goodrich et al. 2013). Therefore, for each subsample we collected 5 mL of sperm adjusted to  $1 \times 10^8$  cells/mL (approximately  $5 \times 10^8$  cells/sample). We obtained ECs from pooled eggs of five females, to reduce male-female compatibility effects that can cause variation in the phenotypic effect of sperm chemoattractants in *M. galloprovincialis* (Evans et al. 2012; Oliver and Evans 2014; Kekäläinen and Evans 2016). Eggs from each female were

adjusted to  $5 \times 10^4$  cells/mL, pooled together, and then left for 1 h to release chemoattractants. The resulting solution was filtered through Whatman filter paper (pore size 11  $\mu\text{m}$ ) to remove eggs, with the filtrated 'egg water' (solution containing chemoattractants) retained for downstream analysis (Oliver and Evans 2014). We added 5 mL of twice-filtered seawater to one sperm subsample from each male (the control samples), and 5 mL of egg water to the other sperm subsample from each male (the treated samples). All samples were incubated at room temperature for 10 minutes, so chemotactic responses could proceed, and then centrifuged for 10 minutes at 4 °C and 2500 rpm (1258 g) to pellet sperm cells from solution. We removed the supernatant, froze the sperm pellets using liquid nitrogen and stored them at -80 °C until required for RNA extractions. We also froze two subsamples of the egg water (EW) solution (i.e. the same egg water source as that added to sperm) and stored them at -80 °C.

#### *RNA extraction and sequencing*

We extracted total RNA from all samples using the RNeasy Plus Universal Mini Kit (Qiagen), based on the manufacturer's instructions, with the following modifications. First, to lyse cells during homogenisation, 100  $\mu\text{L}$  of sterile 0.5 mm glass beads (Daintree Scientific) were added to the samples along with Qiazol<sup>®</sup>, which were then shaken for 45 seconds using a FastPrep<sup>®</sup> 24 benchtop homogeniser (MP Biomedicals). Second, for the phase separation step, samples were transferred to 1.5 mL phase-lock gel heavy tubes (VWR International), avoiding the transfer of any glass beads, and centrifuged at 20,000 g for 30 minutes. Third, we included an on-column DNase digestion step during extraction to avoid DNA contamination (as sperm cells typically contain much more DNA than RNA; Krawetz 2005; Miller et al. 2005). Following extractions, RNA quantity and purity was assessed using a Qubit<sup>®</sup> 2.0 Fluorometer (Invitrogen). An additional assessment of RNA quality was performed by the Australian Genome Research Facility (AGRF), using an Agilent 2100 Bioanalyzer. RNA purified from sperm contain 18S ribosomal RNA (rRNA), but not 28S rRNA (Cappallo-Obermann et al. 2011). Therefore, the presence of 28S RNA provides an indication of potential somatic cell contamination.



**Figure 4.1.** Experimental design. Gonad tissue (G), control sperm (sperm not exposed to egg water; C) and treated sperm (sperm exposed to egg water; T) samples were collected from each of four males (M1-4). Egg water was collected from pooled eggs of five females (see main text); this egg water source was used for all treated samples. Two subsamples of this egg water source were also collected for sequencing. G, C and T samples from M3 and M4, as well as the two egg water subsamples, were sequenced with 100 bp paired-end reads for transcriptome assembly. G, C and T samples from M1 and M2 were sequenced with 50 bp single-end reads to provide additional replicates for differential expression analysis.

Preparation and sequencing of cDNA libraries was performed by AGRF. Libraries were prepared using the Encore Complete RNA-seq Library System (Nugen), which has previously been used for library preparation of sperm RNA in place of standard ribo-depletion techniques (because the majority of ribosomal RNA is highly fragmented in sperm; Sandler et al. 2013; Miller 2014). Sequencing was conducted using an Illumina HiSeq 2500 platform, using the multiplex barcodes provided with the library preparation kit. Gonad tissue, control sperm and treated sperm samples from two males (M3G, M3C, M3T, M4G, M4C, M4T), along with the two egg water samples (EW1, EW2), were sequenced with 100 base pair (bp) paired-end reads for de novo transcriptome assembly and differential expression analysis.

Gonad tissue, control sperm and treated sperm samples from the other two males (M1G, M1C, M1T, M2G, M2C, M2T) were sequenced with 50 bp single-end reads to provide additional power for the differential expression analysis.

#### *De novo transcriptome assembly*

Bioinformatic support for analysing the sequencing data was provided by AGRF. Raw reads were demultiplexed and read quality was subsequently assessed through two methods: (a) using FASTQC (Andrews 2010) with a K-mer size of seven; and (b) by aligning 1000 randomly-selected reads to the non-redundant nucleotide database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Tool n (BLASTN) (BLAST + v2.6.0; Camacho et al. 2009). Trimmomatic (Bolger et al. 2014) was applied prior to transcriptome assembly to remove adapter sequences and low-quality reads, with leading and trailing ends = 5 nucleotides (nt), sliding window = 5 nt and minimum transcripts length = 25 bp.

Transcriptome assembly from read fragments is technically challenging, particularly for organisms likely to contain many novel genes (as expected for *M. galloprovincialis*; Murgarella et al. 2016), and different assembly tools can generate markedly different results (Cerveau and Jackson 2016; Cabau et al. 2017). Here, the approach described by Cerveau and Jackson (2016) was applied to reduce bioinformatic artefacts by combining de novo transcriptome assemblies from different packages. Briefly, independent assemblies were constructed for pooled reads from all paired-end-libraries using two assembler packages, Trinity (Grabherr et al. 2011) and Oases (Schulz et al. 2012). Default parameter settings were used for both assemblers, with the following exceptions: Trinity – number of central processing units (CPUs) = 32, maximum memory = 450 Gb; Oases – minimum transcript length = 200 bp. The two assemblies were clustered to remove redundant transcripts using CD-HIT-EST (Fu et al. 2012), with local sequence alignment (-G 0), sequence identity threshold of 100% (-c 1.00), shorter sequence minimal coverage of 100% (-aS 1.00) and longer sequence minimal coverage of 0.005% (-aL 0.005) (Cerveau and Jackson 2016). Unique transcripts from the two assemblies were then concatenated. Potential protein-coding sequences (CDS) of the unique transcripts were identified using TransDecoder (Haas et al. 2013). Open reading frames containing >100 amino acids were extracted and clustered



again using CD-HIT-EST with 16 threads (-T 16), maximum available memory 16 Gb (-M 16000), local sequence alignment (-G 0), sequence identity threshold of 98% (-c 0.98), shorter sequence minimal coverage of 100% (-aS 1.00) and longer sequence minimal coverage of 0.05% (-aL 0.05). The contigs containing representative potential coding sequences were used as the final transcriptome assembly, and coding sequences retained for downstream annotations. Assembly quality and completeness was estimated with the Benchmarking sets of Universal Single-Copy Orthologs (BUSCO) package v3 (Simão et al. 2015) using the Metazoa database v9.

### *Functional annotation*

As long, non-coding transcripts can contain CDS, the coding potential scores of the assembled transcripts were estimated using FEELnc prior to functional annotation (Wucher et al. 2017), with complete universal single-copy orthologs used as the high-confidence training set in the 'shuffle' training mode. Concurrently, an additional step to control for potential non-target contamination was performed by aligning the assembled transcripts to the NCBI nucleotide database (NT) using BLASTN and removing any significant hits to species that were not invertebrate animals. Non-contaminant transcripts with high coding potential were retained for further annotation. The translated sequences for these transcripts were analysed using InterProScan v5.11-51.0 (Jones et al. 2014; Finn et al. 2017) to classify proteins into families and predict functional domains and sites. Gene Ontology (GO) terms associated with the InterPro entries were also recorded. The minimum sequence length for InterProScan annotation was 200 bp, and the following member databases were searched: ProDom, SMART, ProSiteProfiles, SUPERFAMILY, PANTHER, Pfam and TIGRFAM. Transcripts with high protein-coding potential were also queried against the UniProt (Apweiler 2004), SWISS-PROT (Bairoch and Apweiler 2000) and NCBI non-redundant (NR) protein databases using BLASTX.

### *Differential expression*

The raw reads from both the paired-end and single-end libraries were aligned to the transcriptome using Bowtie v2.3.2 (Langmead and Salzberg 2012), and the alignments were sorted by coordinate in the Sequence Alignment/MAP format using SAMtools v2.3.2 (Li et al. 2009). Read summarisation, i.e. calculation of the raw counts of reads per exon, was

performed with the *featureCounts v1.4.6-p5* program (Liao et al. 2014) of the Subread software package (Liao et al. 2013). As there was considerable between-male variation in sperm transcript profiles, a two-step normalisation of the raw read counts was performed during the differential expression analyses with the packages edgeR (Robinson et al. 2010) and RUVseq (Risso et al. 2014). First, inter-lane normalisation to account for variation in library sizes was conducted using the Upper Quartile (UQ) method (Bullard et al. 2010) in edgeR. Second, to control for additional noise, the RUVr method described by Risso et al. (2014) was used to estimate factors of unwanted variation. To do this, a generalised linear model (GLM) with a negative binomial distribution was fit to the UQ-normalised read counts, including a fixed covariate for the comparison of interest (gonad vs. sperm or control vs. treatment). The matrix of deviance residuals was extracted from the model and a singular value decomposition performed to generate the matrix of unwanted variation (see Risso et al. 2014 for details). The model was then refit with the unwanted variation included as covariates, to simultaneously normalise the read counts and estimate differential expression. Two differential expression analyses were performed: gonad tissue (G) vs. control sperm (C); and control sperm vs. treated sperm (T). In both cases, a batch effect was included to account for the pair-wise experimental design (i.e. samples of each tissue type from the same four males). Adjusted *P*-values were calculated using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) to control the false discovery rate (FDR). Differences in expression were considered to be significant if the FDR-adjusted *P*-value was  $\leq 0.05$  and the log-fold change in expression was  $\geq 2$ . Any transcripts that were present in the egg water samples (i.e.  $\geq 1$  count per million [CPM] in EW libraries) were excluded from the set of differentially expressed genes in the C vs. T comparison.

Functional enrichment of the gene sets was examined using the package TopGO (Alexa and Rahnenfuhrer 2016). Enrichment of Biological Processes GO terms was explored for: (1) the G vs. C differentially expressed genes, to determine gene functions enriched in sperm overall; and (2) the C vs. T differentially expressed genes, to identify sperm gene functions enriched in response to ECs. Functional enrichment in TopGO was performed using Fisher exact tests with the default *P*-value threshold of 0.01.

## 4.4 Results

### *Sequencing, transcriptome assembly and annotation*

We detected 18S and 28S rRNA in gonad tissue samples from all males. In all sperm (control and treatment) samples, 18S rRNA was present, but not 28S rRNA, indicating that our sperm samples were free from somatic cell contamination. We also detected 18S rRNA (but not 28S rRNA) in egg water samples. Following cDNA library construction and sequencing, approximately 213 million reads were generated for the samples sequenced with paired-end reads, and approximately 222 million reads for the samples sequenced with single-end reads (Table 4.1). De novo assembly from the paired-end libraries (eight samples: M3G, M3C, M3T, M4G, M4C, M4T, EW1, EW2; Table 4.1) produced a consensus transcriptome of 159,110 non-redundant transcripts (Table 4.2), with a total assembly size of 105 Mbp (105,280,629 bp). Completeness assessment against the BUSCO metazoan database found 58.5% of complete BUSCOs in the assembly, 30.5% fragmented and 11% missing. This is comparable to expectations of completeness for transcriptomes of highly specialised tissues or cells such as gonads and gametes (e.g. Gaitán-Espitia et al. 2016). It also compares favourably to other molluscan genome and transcriptome assemblies, which are often highly fragmented (Murgarella et al. 2016; Takeuchi 2017).

Using the coding potential scores (CPS) of the training set complete universal single-copy orthologs, FEELnc analysis determined an optimal CPS cut-off of 0.372 for classifying protein-coding transcripts. Application of this cut-off to the assembled transcriptome resulted in the classification of 49,807 transcripts as long non-coding RNA (lncRNA) (31.3% of total transcripts; Table 4.2) and 108,960 transcripts as protein-coding messenger RNA (mRNA) (68.5% of total transcripts; Table 4.2). After filtration of potential contaminant sequences with significant BLASTN hits to organisms that were not invertebrate animals, 108,138 protein-coding transcripts were retained for further analysis (Table 4.2). In total, 53.07% of the assembled coding transcripts had significant matches against at least one database (NR, SwissProt, UniProt, InterPro). Relatively few transcripts matched previously reported genes using BLASTX searches (< 7% for each BLASTX search; Table 4.3); however, identification of protein families and domains using InterProScan was more successful, with 52.18% of transcripts yielding functional information from InterPro (Table 4.3).

**Table 4.1.** Number of raw reads obtained for each *M. galloprovincialis* sample from Illumina sequencing. Paired-end sequencing was used for gonad tissue, sperm control and sperm treated samples from two males (M3 and M4) as well as egg water samples (EW1 and EW2). Single-end sequencing was used for gonad tissue, sperm control and sperm treated samples from the other two males (M1 and M2).

Sample	Group	Read type	Raw reads
M1G	Gonad	50 bp SE	40,997,711
M1C	Sperm control	50 bp SE	34,589,356
M1T	Sperm treated	50 bp SE	38,236,584
M2G	Gonad	50 bp SE	42,321,311
M2C	Sperm control	50 bp SE	33,293,653
M2T	Sperm treated	50 bp SE	32,738,511
M3G	Gonad	100 bp PE	29,330,903
M3C	Sperm control	100 bp PE	23,758,664
M3T	Sperm treated	100 bp PE	25,345,202
M4G	Gonad	100 bp PE	29,207,660
M4C	Sperm control	100 bp PE	25,064,780
M4T	Sperm treated	100 bp PE	23,668,696
EW1	Egg water	100 bp PE	28,813,503
EW2	Egg water	100 bp PE	27,394,096

**Table 4.2.** Summary statistics for the de novo transcriptome assembled from *M. galloprovincialis* gonad, sperm and egg water samples. Following consensus transcriptome construction, numbers of coding and non-coding transcripts were estimated, and potential contaminant transcripts (significant BLASTn hits to organisms that are not vertebrate animals) were filtered from the coding transcripts (see Methods).

Metric	Value
Total transcripts	159,110
N50	723 bp
Mean transcript length	662 bp
Maximum transcript length	14,868 bp
Total non-coding transcripts	49,807
Total coding transcripts	108,960
Non-contaminant coding transcripts	108,138
Mean coding transcript length	675 bp
Coding transcripts N50	737 bp

**Table 4.3.** Annotation statistics for the assembled coding transcripts, based on searches of three protein databases (NCBI NR, SwissProt and UniProt) and the functional database of InterPro. Some transcripts had hits to multiple databases.

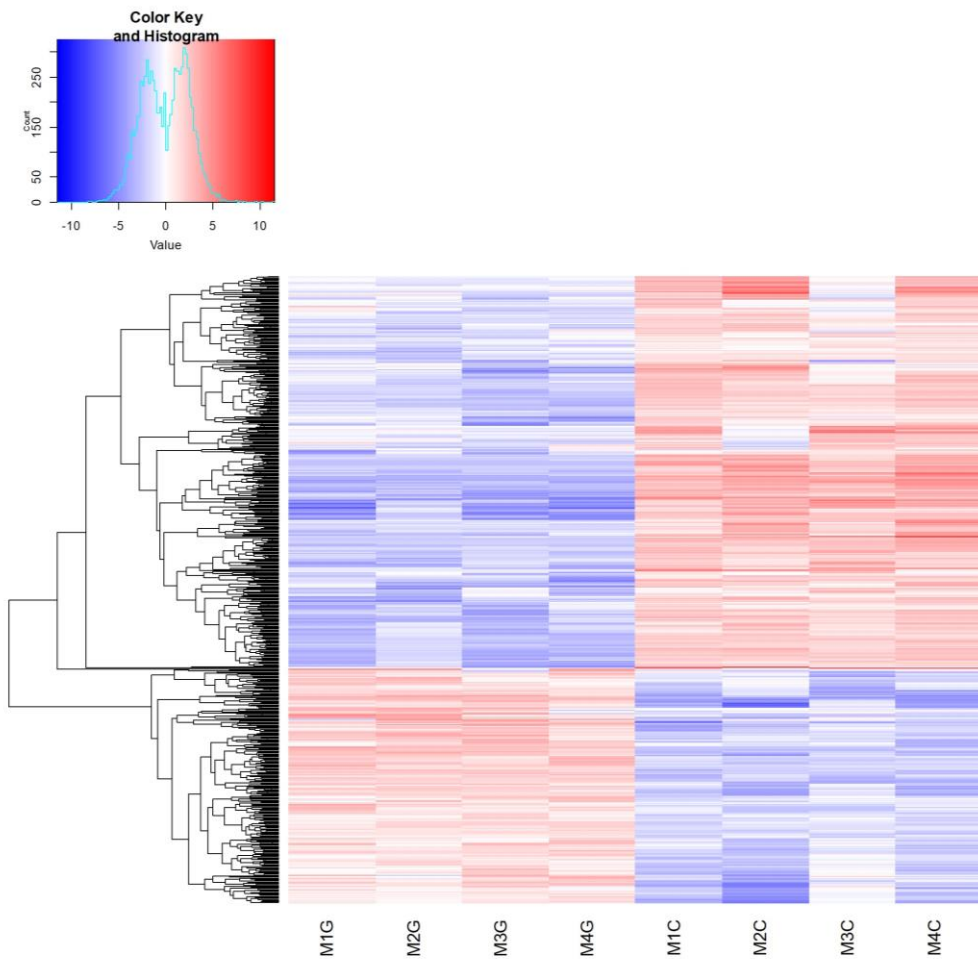
Database (annotation tool)	Number of hits
NR (BLASTX)	7,405 (6.85%)
SwissProt (BALSTX)	1,460 (1.35%)
UniProt (BLASTX)	5,852 (5.41%)
InterPro (InterProScan)	56,421 (52.18%)
At least one database	57,387 (53.08%)
GO terms assigned (InterProScan)	31,903 (29.50%)
Total assembled coding transcripts	103,138

#### *Differential expression: gonad vs. sperm*

A total of 35,856 genes were significantly differentially expressed (FDR-adjusted  $P < 0.05$ ) between gonad tissue and sperm (Fig. 4.2). Of these, 21,190 were downregulated in the sperm samples, while 14,666 were upregulated in the sperm samples. Of the differentially expressed genes between gonad and sperm samples, 3,373 had significant BLASTX hits to the NR, SwissProt or UniProt databases (218 downregulated in sperm and 3154 upregulated in sperm). Using InterProScan, 10,541 differentially expressed genes were assigned functional annotations based on protein families, domains and sites (2,922 downregulated and 7,619 upregulated in sperm). Functional enrichment analysis of the GO categories in this set revealed 40 significantly enriched Biological Processes ( $p < 0.01$ ; Table S4.1). A range of these categories and known proteins have been associated with sperm functions in previous studies (Table 4.4).

#### *Differential expression: control sperm vs. treated sperm*

In the initial comparison of control and treated sperm samples, 1,654 genes were identified as differentially expressed. After subsequent exclusion of all genes present in the egg water samples, 57 genes remained with significant (FDR-adjusted  $P < 0.05$ ) differential expression in the sperm themselves (34 downregulated and 23 upregulated in the treated sperm; Fig. 4.3). Six of the differentially expressed genes had significant BLASTX hits to the NR, SwissProt or UniProt databases, corresponding to five known or predicted functions (Table 4.5). Annotations of protein sites, domains and families were found for 15 differentially expressed genes through InterProScan (eight downregulated and seven upregulated in treated sperm; Table 4.5). The number of functionally annotated genes in the differentially expressed set was too low to detect significant enrichment of GO terms.



**Figure 4.2.** Heatmap of the 35,856 transcripts that were differentially expressed (FDR-adjusted  $P$ -value  $< 0.05$ ) between gonad tissue and sperm. Gonad tissue (G) and sperm samples (C), collected from each of four males (M1-M4), are shown in columns, and hierarchically-clustered transcripts are shown in rows. Expression values (following RUVr normalisation; see Methods) are  $\log_2$  transformed and centred by the median for each transcript.

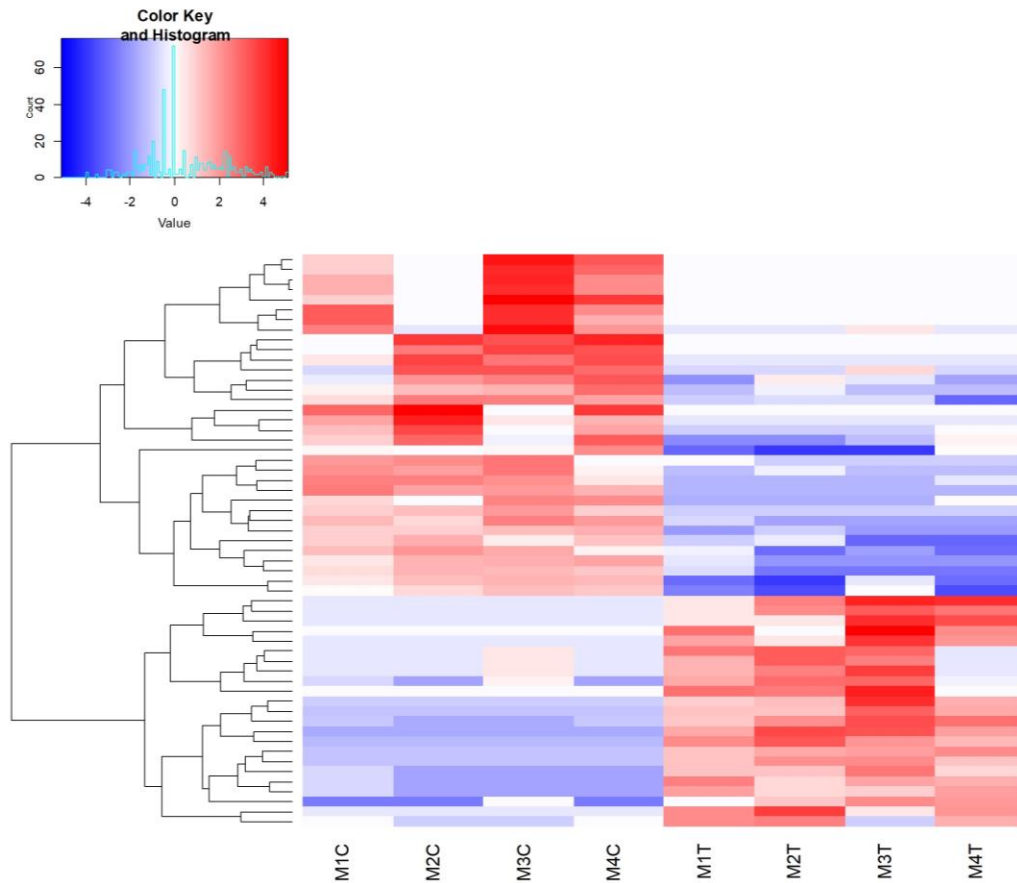
**Table 4.4.** Selected gene ontology categories enriched in the gonad vs sperm differentially expressed set, which have been linked to sperm-specific functions. For a full list of enriched gene ontology categories in the gonad vs. sperm comparison, see Appendix 1.

GO category	Regulation in sperm	Hits to known proteins	Possible sperm functions
Translation (GO:0006412)	Upregulated	40S and 60S RPs, 39S mitochondrial RP, proteasome subunits	Paternal control of embryo development <sup>1</sup> , capacitation <sup>2</sup> , sperm motility <sup>3</sup>
Microtubule-based process (GO:0007017)	Upregulated	Tubulin chains (alpha, beta, epsilon)	Sperm motility <sup>4</sup>
Protein folding (GO:0006457)	Upregulated	TCP1 subunits, GRP94, calreticulin, HSP90	Sperm motility and capacitation <sup>5,6,7</sup>
Regulation of transcription, DNA-templated (GO:0006355)	Both	<u>Upregulated</u> : Crc4-Not subunit, RFX proteins, estrogen receptor, Elk-3, FOX proteins, KAT2B, histone sap130 deacetylase, MAD, MyRF, NF-kappaB, NR subfamilies, CBFA2T1, Sbn0, SF3B IIIB subunit, XBP, YBP <u>Downregulated</u> : FOX protein G1, Pax-6, AP-2 epsilon	Paternal regulation of embryo development <sup>8</sup>
Tricarboxylic acid cycle (GO:0006099)	Upregulated	SDH (IP subunit), ACO2, IDH3G	Sperm motility <sup>9,10</sup>
Small GTPase-mediated signal transduction (GO:0007264)	Upregulated	Rab proteins, Ras proteins, ADP ribosylation factors	Sperm capacitation <sup>11</sup>
Actin filament bundle assembly (GO:0051017)	Upregulated	Actn	Acrosome reaction <sup>12,13</sup>
Actin crosslink formation (GO:0051764)	Upregulated	Actn	Acrosome reaction <sup>12,13</sup>
Microtubule anchoring at centrosome (GO:0034454)	Upregulated	No known protein hits	Fusion of sperm and egg pronuclei <sup>14</sup>
Protein localisation to centrosome (GO:0071539)	Upregulated	No known protein hits	Fusion of sperm and egg pronuclei <sup>14</sup>



GO category	Regulation in sperm	Hits to known proteins	Possible sperm functions
mRNA splicing, via spliceosome (GO:0000398)	Majority upregulated	SNW1, PRPF proteins, U2AF subunit	Paternal regulation of embryo development <sup>15</sup>
Protein phosphorylation (GO:0006468)	Majority upregulated	Aky, RAC, CSKN1, DYRK1A, SKR2, CDK proteins, Tnik, pkaC, SRPK proteins, CASK, IRAK1, GSK3B, Tssk4, ACVR1, MAPK, EKRT2	Capacitation <sup>16,17,18</sup> and sperm motility <sup>19,20</sup>
Transmembrane receptor protein tyrosine kinase (GO:0007169)	Upregulated	No known protein hits	Capacitation <sup>21,22</sup>

1. Fischer et al. (2012); 2. Gur and Breitbart (2006); 3. Inaba et al. (1998); 4. Bhagwat et al. (2014); 5. Nakamura et al. (1993); 6. Huang et al. (1999); 7. Dun et al. (2011); 8. Hammoud et al. (2009); 9. Peterson and Freund (1970); 10. Qiu et al. (2016); 11. Tulsiani and Abou-Haila (2004); 12. Tilney et al. (1973); 13. Tilney et al. (1987); 14. Simerly et al. (1995); 15. Sandler et al. (2013); 16. Visconti and Kopf (1998); 17. Breitbart and Naor (1999); 18. Aravindan et al. (2012); 19. Tash and Means (1982); 20. Tash and Means (1983); 21. Osheroff et al. (1999); 22. Visconti et al. (1999).



**Figure 4.3.** Heatmap of the 57 transcripts that were differentially expressed (FDR-adjusted  $P$ -value < 0.05) between sperm control and treated samples. Control (C) and treated (T) sperm samples, collected from each of four males (M1-M4), are shown in columns, and hierarchically-clustered transcripts are shown in rows. Expression values (following RUVr normalisation; see Methods) are  $\log_2$  transformed and centred by the median for each transcript.

**Table 4.5.** Functional annotations for differentially expressed genes in the sperm control vs. sperm treated comparison, i.e. following exposure of sperm to egg chemoattractants. LogFC = log fold change from sperm control to sperm treated; *P* = false discovery rate-adjusted *P*-value.

Transcript	LogFC	<i>P</i>	InterPro annotation	GO terms	Known protein hits
<i>Downregulated in response to egg chemoattractants</i>					
TRINITY_1961799	-6.08	0.023	Adenylyl cyclase class-3/4/guanylyl cyclase domain	GO:0016849, GO:0009190, GO:0035556	GUCY2C
TRINITY_17652	-5.62	0.022	Cation-independent mannose-6-phosphate receptor repeat	GO:0006810, GO:0016021, GO:0005737, GO:0005215	
TRINITY_21687	-5.61	0.023	Coagulation factor 5/8 C-terminal domain	GO:000551	
TRINITY_942076	-5.55	0.043	GPI mannosyltransferase family	GO:0016757, GO:001675	
TRINITY_1633452	-5.45	0.032	MAM domain	GO:0016020, GO:001602	
TRINITY_1652321	-4.51	0.023	Runt domain	GO:0003677, GO:0006355, GO:0003700	
TRINITY_1612353	-4.24	0.032	Dynein heavy chain family	GO:0007018, GO:0030286, GO:0003777	Dynein beta chain, ciliary
TRINITY_656958	-3.83	0.029	WD40 repeat	GO:000551, GO:0005515	CIAO1 homolog (predicted)
<i>Upregulated in response to egg chemoattractants</i>					
TRINITY_1604106	4.09	0.033	Heavy metal-associated domain	GO:0046872, GO:0030001	
TRINITY_1618621	4.09	0.027	PDZ domain	GO:000551, GO:0005515	
TRINITY_1858828	4.21	0.047			FCHSD2-like isoform
TRINITY_485836	4.86	0.023	Death effector domain	GO:0005515, GO:0042981	
TRINITY_915778	5.63	0.042			CFAP54 (predicted)
TRINITY_670085	5.94	0.021	Cadherin domain	GO:0016020, GO:0005509, GO:0007156	
TRINITY_1508387	6.13	0.037	Peptidase M20 family	GO:0008152, GO:0016787	
TRINITY_1358722	6.33	0.027	Dynein heavy chain family	GO:0007018, GO:0003777	
TRINITY_926890	6.66	0.024	PDZ domain	GO:000551, GO:0005515	

## 4.5 Discussion

Here, we have identified differential gene expression as a putative mechanism underlying phenotypic responses by sperm to chemical signals released by eggs. Our findings therefore challenge the prevailing view that mature sperm are transcriptionally silent (Hecht 1998), despite the emerging evidence from model organisms that they contain distinct, functional populations of RNAs (Dadoune 2009; Hosken and Hodgson 2014). Specifically, we show that *M. galloprovincialis* sperm carry many transcripts that are significantly enriched compared to gonad tissue, indicating they are not merely remnants of spermatogenesis. Furthermore, we show that expression levels of multiple genes are altered when sperm are exposed to ECs, and identify several putative protein functions in this gene set relevant to the phenotypic effects of sperm chemotaxis. These findings included both upregulation and downregulation of genes in response to chemoattractants. This suggests that: (a) transcription can be activated or promoted by egg-derived signals; and (b) there may be a background level of transcription in sperm, which is dampened for certain genes (i.e. the significantly downregulated genes) in response to chemoattractants. Together, our results provide the first direct evidence of differential gene expression in mature, ejaculated sperm in any organism.

We detected nearly 15,000 protein-coding transcripts that were enriched in sperm samples compared to gonads, which are likely to be of functional significance to mature sperm rather than simply reflections of spermatogenesis. Recent studies have identified many thousands of mRNA transcripts in mature sperm in several taxa, including mammals (Ostermeier et al. 2002; Zhao et al. 2006; Butts et al. 2012; Das et al. 2013; Selvaraju et al. 2017), *Drosophila melanogaster* (Fischer et al. 2012) and *Caenorhabditis elegans* (Ma et al. 2014). These sperm RNA profiles have been correlated with differences in fertility, and protein functions identified that are relevant to sperm activity or embryo development (Dadoune 2009; Jodar et al. 2013). In the present study, although overall rates of hits against known genes were relatively low (as is typical for mussels and other molluscs; Saavedra and Bachère 2006; Takeuchi 2017), we detected a range of functional categories that were differentially expressed in sperm and gonads. Notably, several sperm-upregulated functions were involved in the regulation of transcription and translation; many of these mRNAs are likely to be passed into fertilised eggs to mediate embryo development (Fischer et al. 2012). Similarly, two sperm-upregulated categories corresponded to organisation of the centrosome, which is passed into fertilised eggs and crucial for fusion of sperm and egg pronuclei (Simerly et al. 1995). We also found sperm-enriched categories that are likely to be involved in

functions prior to zygote formation, such as sperm capacitation (e.g. GTPase-mediated signal transduction and protein phosphorylation; Visconti and Kopf 1998; Breitbart and Naor 1999; Tulsiani and Abou-Haila 2004) and acrosome reaction (actin bundle organisation; Tilney et al. 1973, 1987). The enrichment of such transcripts suggests sperm RNA populations may be functionally important both before and after fertilisation.

Our finding that a distinct set of protein-coding transcripts was differentially expressed in sperm after exposure to egg water suggests that these genes may influence sperm chemotaxis responses. We identified a range of functional protein categories relevant to sperm motility and chemotactic movement. One of the upregulated genes matched to protein domains that occur in guanylyl cyclases (GCs); membrane-bound GCs have been identified in sea urchin sperm as the receptor for chemoattractant peptides, which initiates the chemotaxis signalling cascade (Matsumoto et al. 2003; Kaupp et al. 2006). Structural changes to the plasma membrane are unlikely to occur in mature sperm, and the precise cellular location of the downregulated gene is not clear. However, gene ontology terms for this transcript include phosphorous-oxygen lyase activity, which is an important process in the activation/deactivation of membrane-bound GCs (Pichlo et al. 2014). Therefore, one possibility is that the regulation of this transcript is involved in the maintenance of receptor sensitivity to chemoattractants. Other protein categories that may be involved in sperm motility signalling pathways include the upregulated PDZ domain (which occurs in adaptor molecules that transmit signals in sperm flagella; Fujita et al. 2000) and peptidase M20 family (which occurs in enzymes that regulate protein 14-3-3, a molecule linked to sperm motility; Huang et al. 2004; Lalle et al. 2011). One downregulated transcript matched WD-40 repeat-containing proteins, which have been found in sperm flagella and may be physiologically altered during chemoattraction (Hozumi et al. 2008). However, this gene had a significant BLASTX hit as a probable cytosolic iron-sulfur assembly protein homolog (CIAO1), which has not yet been directly linked to sperm function. Finally, some differentially expressed transcripts that matched the dynein heavy chain protein family (one upregulated and one downregulated transcript) and predicted cilia- and flagella-associated protein 54 (CFAP54), which are important for the structure and motor activity of sperm flagella (Inaba 2003; Rashid et al. 2006; McKenzie et al. 2015; Hu et al. 2017). Again, major structural changes to the flagella are unlikely to occur in mature sperm, but it is possible that these genes are involved in signalling pathways that control flagellar motor activity. Together, these results suggest a variety of gene functions that may underlie chemotactic motility changes in sperm.

We also identified a number of protein functions in differentially expressed genes that are related to the acrosome reaction and changes of the cell surface for sperm-egg interaction (capacitation), both of which are known responses to ECs (Kekäläinen and Evans 2016). One upregulated gene contained a cadherin domain, which is found in proteins that control calcium-dependent cell-cell adhesion and have been linked to sperm-egg fusion (Goodwin et al. 2000; Marín-Briggiler et al. 2010; Caballero et al. 2014). Amplified expression of this gene may represent female-induced preparation of the sperm for fusion with the egg. Downregulated protein categories included a cation-independent mannose-6-phosphate receptor (CI-MPR) repeat and an MAM domain; both of these occur in cell surface glycoproteins, which undergo extensive reorganisation when sperm encounter ECs (Kekäläinen and Evans 2016). Studies on mammalian systems have detected CI-MPR proteins in the acrosome of mature sperm (Tsuruta and Brien 1995; Tsuruta et al. 2000) and linked them to the recruitment of acrosomal enzymes (Aguilera et al. 2016). Furthermore, there was decreased expression of a gene matching to the protein family of glycosylphosphatidylinositol (GPI) mannosyltransferase, a group of enzymes that manufacture GPI glycolipids for anchoring other proteins to cell surfaces (Oriol et al. 2002). In sperm, GPI-anchored proteins are released from the cell surface during the acrosome reaction and lipid raft reorganisation in preparation for fertilisation (Kondoh et al. 2005; Watanabe and Kondoh 2011). Therefore, downregulation of genes associated with the CI-MPR, MAM and GPI mannosyltransferase protein categories may reflect changes to the cell surface that occur during capacitation and the acrosome reaction, which are initiated by ECs (Kekäläinen et al. 2015).

Several protein functions were identified in the differentially expressed genes that might reflect previously unidentified responses to ECs. Upregulated genes included significant hits to a death effector domain (DED) and heavy metal associated (HMA) domain, which may be involved in promoting sperm survival and motility. Proteins containing DED are involved in the regulation of apoptosis, both through activation and inhibition, and apoptosis inhibitors are likely to be important for survival of mature sperm (Cayli et al. 2004). There is also some evidence that heavy metal-binding proteins might prolong sperm motility in broadcast spawners (Johnson and Epel 1983). Several downregulated genes also suggested novel responses to chemoattractants, including a gene that matched a coagulation factor V/VIII C-terminal domain. Studies have reported coagulation factors, which are typically involved in blood clot formation, in human seminal plasma and linked them to post-ejaculation semen coagulation (Matsuda et al. 2002). In

*M. galloprovincialis*, semen form clots shortly after spawning, possibly as a means of avoiding sperm dilution and limitation (Torrado et al. 2003). This may be linked to a seminal protein described by Torrado et al. (2003) as male-associated peptide, which exhibits similar esterase activity to known coagulation factors (Zur and Nemerson 1978). Therefore, it is possible that downregulation of the protein we identify here is involved in the release of sperm from clots upon detection of chemoattractants. Lastly, another downregulated gene matched runt domains, which are an important group of transcriptional regulators that can both activate and repress expression of downstream targets (Wheeler et al. 2000). One possibility is that this gene is involved in regulating the expression of other proteins involved in sperm chemotactic responses. Alternatively, runt domain proteins often play important roles in embryo development (Coffman et al. 1996; Canon and Banerjee 2000), and downregulation of this gene may represent changes in paternal transcription factors that are passed into fertilised eggs. These functional annotations suggest a variety of novel responses to ECs that could be fruitful avenues for experimental investigation.

Based on the findings presented here, we suggest two main areas for further elucidation of the molecular mechanisms of differential sperm chemotaxis. First, we need to investigate whether the extent of differential gene expression in treated and untreated sperm is dependent on male-by-female effects. We predict that the extent of up- and downregulation of these genes will depend on the specific identity of egg chemoattractant donor females, thus providing much needed mechanistic insight into the previously reported intraspecific variation in sperm chemotaxis (Evans et al. 2012; Oliver and Evans 2014; Kekäläinen and Evans 2016). Second, we require studies that identify the precise egg-derived chemical signals that induce differential gene expression and chemotactic responses by sperm. The presence of RNA in our egg water samples raises the intriguing possibility that RNA released by the eggs themselves might play a regulatory function during sperm gene expression. Although research into potential functions of extracellular RNA is in its infancy, there is growing evidence that RNAs can be transferred between cells (Dinger et al. 2008) and even between species (Kim et al. 2014). Intriguingly, micro RNAs (miRNAs), which typically regulate gene expression, have been detected in cell-secreted vesicles of equine ovarian fluid (da Silveira et al. 2012). It would be interesting to determine whether the RNA transcripts found in egg water in the present study are adaptively exuded signals released by the egg to manipulate the sperm, or instead merely transcripts that have leaked from the eggs during sample collection. Our experiment focused on protein-coding mRNA and was not designed to capture

small RNAs (e.g. miRNAs or small interfering siRNAs), which may be important components of extracellular signalling pathways (Dinger et al. 2008; Patton et al. 2015; Quesenberry et al. 2015). We therefore hope that the present study will lead to detailed investigations of egg-water enriched RNAs in order to better understand the mechanisms that control egg-sperm chemical signalling.

In conclusion, we present the first empirical evidence for differential gene expression in sperm after ejaculation, raising fundamental questions about whether mature sperm are capable of altering gene expression in a broader range of species and scenarios. Theoretical predictions suggest that suppressing transcription in sperm cells should benefit males by avoiding within-ejaculate competition and haplo-diploid conflict (Haig and Bergstrom 1995; Immler 2008; Hosken and Hodgson 2014). This may partly explain the drastic remodelling of sperm chromosomes that occurs in late spermatogenesis (Hecht 1998). However, our results suggest the possibility that limited expression of sperm genes could have important implications for sperm function and reproductive success. Interestingly, human sperm DNA retains normal histones at specific sites in the genome (Gatewood et al. 1987), which could leave some sequences available for transcription. Although the *M. galloprovincialis* genome is currently not well characterised (Murgarella et al. 2016), it would be fascinating to compare patterns of sperm DNA packaging to the transcript sequences that we identify as differentially expressed in response to chemoattractants. We anticipate that the possibility of gene expression in sperm cells will open many novel avenues of research into the control of sperm behaviour and function.

#### **4.6 Acknowledgements**

We thank Cameron Duggin for assistance with mussel collections, Sherralee Lukehurst, Samuel Lymbery, Robert Dugand, Natalie Rosser, Phillip Allen and Angela Eads for practical help, Joanito Liberti and Ryan Dosselli for assistance in developing the RNA extraction protocol, and Blair Bentley for advice on analyses. The Australian Genome Research Facility provided sequencing and bioinformatic support, for which we particularly thank Juan Montenegro, Sonika Tyagi, Matthew Tinning and Saurabh Shrivastava. Funding was provided by the UWA School of Animal Biology (RAL) and the Australian Research Council (JPE; Grant Numbers DP150103266 and DP170103290). RAL was supported by the Hackett Postgraduate Research Scholarship and the Bruce and Betty Green Postgraduate Research Top-Up Scholarship.



## 4.7 Supplementary Materials

### Supplementary Tables

**Table S4.1.** All gene ontology categories significantly enriched in the differentially expressed set of genes between gonad tissue and sperm.

GO ID	Term	Total genes	DE genes	<i>P</i>	Regulation in sperm
GO:0015074	DNA integration	729	431	< 0.001	Downregulated
GO:0006412	translation	374	180	< 0.001	Upregulated
GO:0007017	microtubule-based process	1312	447	< 0.001	Upregulated
GO:0006457	protein folding	107	74	< 0.001	Upregulated
GO:0051603	proteolysis involved in cellular protein catabolic process	231	121	< 0.001	Upregulated
GO:0006355	regulation of transcription, DNA-templated	811	340	< 0.001	Both
GO:0006099	tricarboxylic acid cycle	35	26	< 0.001	Upregulated
GO:0006913	nucleocytoplasmic transport	89	51	< 0.001	Upregulated
GO:0006165	nucleoside diphosphate phosphorylation	75	40	< 0.001	Upregulated
GO:0006183	GTP biosynthetic process	25	20	< 0.001	Upregulated
GO:0006228	UTP biosynthetic process	25	20	< 0.001	Upregulated
GO:0006241	CTP biosynthetic process	25	20	< 0.001	Upregulated
GO:0006310	DNA recombination	270	111	< 0.001	Majority downregulated
GO:0007264	small GTPase mediated signal transduction	367	146	< 0.001	Majority upregulated
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	13	11	< 0.001	Upregulated
GO:0006511	ubiquitin-dependent protein catabolic pr...	205	99	< 0.001	Upregulated
GO:0006139	nucleobase-containing compound metabolic. process	3544	1438	< 0.001	Upregulated
GO:0006886	intracellular protein transport	305	132	< 0.001	Majority upregulated
GO:0015992	proton transport	67	37	< 0.001	Upregulated
GO:0015986	ATP synthesis coupled proton transport	30	20	< 0.001	Upregulated
GO:0022900	electron transport chain	20	16	< 0.001	Majority upregulated
GO:0046034	ATP metabolic process	94	52	< 0.001	Upregulated
GO:0032775	DNA methylation on adenine	14	11	0.001	Downregulated
GO:0042176	regulation of protein catabolic process	11	9	0.001	Upregulated
GO:0051017	actin filament bundle assembly	10	9	0.001	Upregulated
GO:0007009	plasma membrane organisation	19	11	0.001	Upregulated
GO:0051764	actin crosslink formation	6	6	0.001	Upregulated
GO:0006334	nucleosome assembly	29	18	0.001	Majority upregulated
GO:0045454	cell redox homeostasis	66	34	0.002	Upregulated

GO ID	Term	Total genes	DE genes	<i>P</i>	Regulation in sperm
GO:0006662	glycerol ether metabolic process	8	7	0.003	Upregulated
GO:0051607	defence response to virus	120	55	0.003	Downregulated
GO:0034454	microtubule anchoring at centrosome	12	9	0.004	Upregulated
GO:0071539	protein localisation to centrosome	12	9	0.004	Upregulated
GO:0006631	fatty acid metabolic process	38	14	0.004	Majority upregulated
GO:0000398	mRNA splicing, via spliceosome	97	42	0.005	Majority upregulated
GO:0006270	DNA replication initiation	55	28	0.005	Upregulated
GO:0006468	protein phosphorylation	878	327	0.005	Majority upregulated
GO:0006094	gluconeogenesis	21	13	0.007	Upregulated
GO:0007169	transmembrane receptor protein tyrosine kinase signalling pathway	17	11	0.007	Upregulated
GO:0006414	translational elongation	17	11	0.008	Majority upregulated

## CHAPTER FIVE

### **Multivariate sexual selection on ejaculate traits under sperm competition**

*Accepted for publication in The American Naturalist.*

*This chapter is presented in its accepted form, apart from minor changes to formatting and referencing for consistency with previous and subsequent chapters.*



## 5.1 Abstract

The widespread prevalence of sperm competition means that ejaculates face intense sexual selection. However, prior investigations of sexual selection on gametes have been hampered by two difficulties: (1) deriving estimates of relative fitness from sperm competition trials that are comparable across rival male and female genotypes; and (2) obtaining measures of competitive fertilisation success that are not confounded by post-zygotic effects. Here, we exploit the experimental tractability of a broadcast spawning marine invertebrate to overcome these challenges and characterise multivariate sexual selection on sperm traits when multiple ejaculates compete. In multi-male spawning events, we tracked real-time success of sperm using fluorescent tags that are visible inside fertilised eggs. We then used multivariate selection analyses to identify patterns of linear and non-linear sexual selection on multiple sperm morphology and motility traits. Specifically, we found non-linear selection against divergent combinations of sperm length, velocity and swimming path linearity. These patterns likely reflect the way different swimming strategies allow sperm to locate and track eggs. Our results demonstrate that there are overall patterns of selection on ejaculates across a biologically realistic range of ejaculate-ejaculate and ejaculate-female interactions; therefore, there is the potential for adaptive evolution of ejaculate traits under sperm competition.

## 5.2 Introduction

Since Parker (1970) introduced the concept of sperm competition (competition for fertilisation among ejaculates of multiple males) in insects, considerable evidence has accumulated to show that the phenomenon is taxonomically pervasive (Birkhead and Møller 1998; Simmons 2001; Evans and Sherman 2013). Consequently, in most sexually reproducing species, ejaculates face the pressures of both outcompeting rival sperm, and negotiating female mechanisms that can selectively bias fertilisations toward a subset of males (cryptic female choice; Eberhard 1996). Understanding how these selective forces target ejaculate traits has become a major focus of both theoretical (reviewed by Parker 1998) and empirical studies (reviewed by Simmons and Fitzpatrick 2012). Accordingly, several studies have reported an association between the relative numbers of sperm in competing ejaculates and competitive fertilisation success, thus supporting raffle-based models of sperm competition (see reviews by Simmons 2001; Parker and Pizzari 2010). However, conflicting findings for many traits (e.g. size, shape and motility patterns of sperm cells; see Simmons and Fitzpatrick 2012; Fitzpatrick and Lüpold 2014), coupled with an increased awareness of the functionally integrated nature of multiple sperm and ejaculate traits, indicate that we have not yet accounted for the complexities of selection acting on ejaculates (Pizzari and Parker 2009).

In addition to the need to account for the complex patterns of selection that act on ejaculates, two further factors have hampered our ability to characterise sexual selection on ejaculate traits. First, due to practical constraints, a male's competitive fertilisation success is typically inferred from his paternity share of resultant offspring rather than at the moment of conception (Birkhead et al. 2004). This relies on the assumption that the ratio of surviving offspring from each competing male is equivalent to the ratio of eggs fertilised by each male's sperm (García-González 2008a). However, paternity success can also reflect differential embryo viability, which can be influenced by a range of genetic and non-genetic post-zygotic effects (Gilchrist and Partridge 1997; Tregenza et al. 2003; García-González and Simmons 2005; Crean et al. 2013). Therefore, paternity share may not accurately reflect competitive fertilisation success (Olsson et al. 1999; Birkhead et al. 2004; García-González and Simmons 2007; García-González 2008a). Recent approaches in some internally-fertilising systems have made progress in overcoming this problem. For example, in sperm competition trials using breeding lines of zebra finches with divergent sperm lengths, Bennison et al. (2015) quantified competitive success by estimating the proportion of long and short sperm trapped in egg membranes. Furthermore, recent studies in some invertebrates have developed transgenic lines expressing fluorescent proteins in all cells (including sperm) (Manier et

al. 2010; Marie-Orleach et al. 2014). In *Drosophila melanogaster*, these lines have been used to (a) separate competitive fertilisation success in eggs from adult paternity estimates (Droge-Young et al. 2012), and (b) quantify the relationship between multiple ejaculate traits and sperm competitive success at different post-mating stages (Lüpold et al. 2012). Nevertheless, the difficulties in applying such techniques in most systems, particularly those requiring the establishment of breeding lines, mean that relative paternity share is still the most common measure of competitive success.

A second challenge facing attempts to characterise selection on ejaculate traits is the growing recognition that individual sperm competition trials may not provide realistic measures of population-level relative fitness (García-González and Evans 2011). In most study systems, double (dyadic) mating trials are commonly used to estimate sperm competitiveness, for example by measuring the proportion of offspring sired by the second of two males to mate with a female ( $P_2$ ; Boorman and Parker 1976; García-González 2008a). However, such estimates from single mating trials (two males, one female) will be dependent on variation in: (a) the competitive ability of rival male ejaculates and ejaculate-by-ejaculate genotypic interactions (Clark et al. 2000; García-González 2008b; García-González and Evans 2011; Engqvist 2013); and (b) ejaculate-by-female genotypic interactions (Clark and Begun 1998; Clark et al. 1999). These context-specific effects on sperm competition outcomes are interesting in themselves (Bjork et al. 2007), particularly for internal fertilisers where processes such as sperm displacement mean that competitive interactions can be restricted to ejaculates from two males competing within a single female (Lefevre and Jonsson 1962; Simmons et al. 1999; Manier et al. 2010; but see Zeh and Zeh 1994). However, population-level patterns of selection on ejaculates will usually depend on the relative reproductive fitness of males across many matings involving different females and rival males (García-González and Evans 2011). Moreover, in the case of external fertilisers, even a single mating event might involve gametes from many males and females (Levitan 2010). We therefore require techniques that provide more complete estimates of population-level relative fitness to estimate selection on ejaculates.

The externally fertilising mussel, *Mytilus galloprovincialis*, is an emerging model system in post-ejaculatory sexual selection research and ideally suited for estimating selection on ejaculates. *M. galloprovincialis* forms large aggregations, and multiple individuals spawn synchronously during reproductive events (Wilson and Hodgkin 1967; Seed 1976), meaning that ejaculates are likely to

face intense sperm competition. Importantly, we now have a method that enables us to measure competitive fertilisation success at the moment of conception in this species (Chapter 2). As with many other bivalve molluscs, mussel embryos inherit mitochondria from both parents through the process of doubly uniparental inheritance (Zouros et al. 1994; Zouros 2013). This allows us to label the sperm mitochondria of ejaculates from individual males and track their real-time fertilisation success when in competition with multiple ejaculates (by recording eggs with labelled mitochondria). This method avoids potentially confounding post-zygotic processes that may differentially affect embryo survival. Moreover, in combination with the experimental tractability of broadcast spawners (e.g. Evans and Marshall 2005; Fitzpatrick et al. 2012; Johnson et al. 2013; Eads et al. 2016b; Monro and Marshall 2016), this tool provides the potential to derive meaningful measures of male competitive fitness in biologically realistic multi-male spawning events.

Here, we combine the experimental tractability of *Mytilus galloprovincialis* with the powerful tools of multivariate selection analyses (Lande and Arnold 1983; Phillips and Arnold 1989; Blows and Brooks 2003) to characterise multivariate sexual selection on ejaculate traits when sperm from multiple males compete for fertilisations. We use fluorescent mitochondrial dyes to track the competitive success of ejaculates from individual males in competition with multiple rival ejaculates, enabling us to account for variation in rival competitiveness. Moreover, our competitive trials involve egg pools from multiple females to account for variation in female effects or ejaculate-by-female interactions. Therefore, we provide biologically meaningful estimates of reproductive fitness that are comparable across competitive scenarios. We then apply multivariate statistical methods for estimating patterns of linear (i.e. directional) and non-linear (e.g. stabilising, disruptive or correlational) sexual selection on multiple sperm morphology and motility traits. In doing so, we test how selection under sperm competition acts on a range of potentially interacting ejaculate traits.

### **5.3 Methods**

#### *Collection and spawning*

*Mytilus galloprovincialis* is a gonochoristic, broadcast spawning mussel that inhabits temperate and sub-polar intertidal regions in both the Northern and Southern Hemispheres (Daguin and Borsa 2000), including temperate Australian latitudes (Westfall and Gardner 2010). Mussels in this genus form dense aggregations on intertidal substrates, and natural populations undergo mass, synchronised spawning events that result in high local gamete densities from multiple individuals

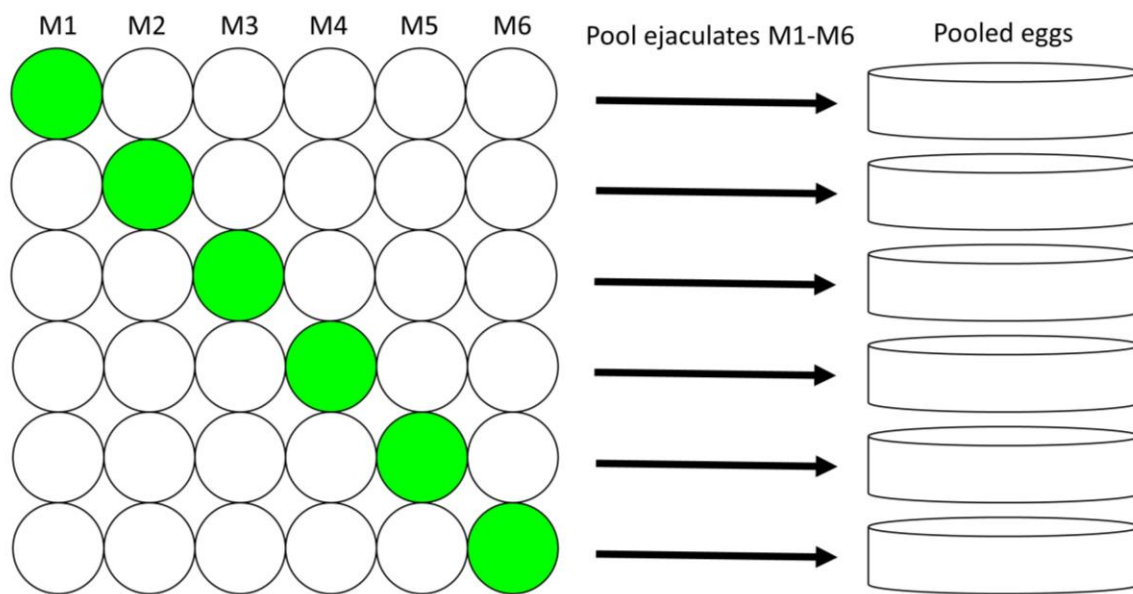


(Wilson and Hodgkin 1967; Villalba 1995; Baur 1998; Gosselin 2004). We collected adult mussels from Woodman Point, Western Australia (32°14' 03.6"S, 115°76' 25"E) during the 2016 reproductive season (June-September) and housed them in tanks of recirculating filtered seawater (FSW) at the University of Western Australia. Mussels were used for experimental trials within 1-2 weeks of collection. Spawning was induced by increasing the temperature of water baths from ambient to 28°C (Chapter 2). Immediately upon spawning (when the sex of individuals was determined), we removed individuals from the water bath, washed them to remove contaminating gametes, and placed them in separate 250 mL cups covered by FSW. When gamete concentrations were suitably high (within 30 minutes of spawning), we removed mussels from the spawning cups and estimated gamete concentrations using an improved Neubauer haemocytometer for sperm (subsamples fixed with 1% formalin) and cell counts in a known volume for eggs. These were then adjusted to the required concentrations for fertilisations and sperm trait measurements as described below. Sperm in *Mytilus* spp. can remain fertilisation-competent for longer than 11 hours (Sprung and Bayne 1984), and studies in *M. galloprovincialis* have found sperm remain fully motile after 3-hour trials (Evans et al. 2012; Oliver and Evans 2014).

### *Experimental design*

Our experiment was designed to obtain robust measures of competitive fertilisation success in realistic multi-individual spawning events. Moreover, we aimed to ensure our fitness measure (competitive fertilisation success) was comparable across males and blocks (see below) and accurately represented relative offspring share in the next generation (which can be challenging for reproductive success estimates in selection analyses; M. Morrissey pers. comm.; see Supplementary Methods for details). We measured competitive fertilisation success of each male's ejaculate within replicate 'populations' of competitors (see Evans and Garcia-Gonzalez 2016 for a review of studies using similar designs to estimate relative reproductive fitness). Each population (hereafter referred to as 'block') contained sperm from six males (Fig. 5.1; n = 20 blocks, 120 males total) competing to fertilise eggs pooled from six to eight females. Pooling eggs from multiple females provided estimates of male competitive fertilisation success that account for variation in male-by-female interactions, which are typical of fertilisations in *M. galloprovincialis* (Evans et al. 2012; Oliver and Evans 2014). We determined that six males per block were sufficient to provide a representative sample of overall variation in sperm fertilising ability of the background population, using simulations from data previously collected in our laboratory on fertilisation success of *M. galloprovincialis* males (Fitzpatrick et al. 2012; for details

of simulations see Supplementary Methods; Tables S5.1, S5.2). We individually counted fertilised eggs for each male under standardised counting conditions (described below), and estimated competitive fertilisation success as the proportion of fertilisations attributable to each male over all fertilised eggs in the block (i.e. controlling for any stochastic temporal variation in egg ‘ripeness’ across blocks). Our design therefore provided estimates of competitive fertilisation success that were comparable across blocks; confirmed by the absence of significant between-block variation in competitive fertilisation success (Supplementary Methods; Table S5.3).



**Figure 5.1.** Overview of a single block of the experimental design used to estimate competitive fertilisation success. Each block involves ejaculates from each of six males (M1-M6) competing to fertilise eggs pooled from at least six females. Six competitive fertilisation assays are performed in each block; in each assay sperm from a different focal male is labelled with MitoTracker Green while the other males’ ejaculates are left unlabelled. Competitive fertilisation success is measured for each male by counting the number of eggs (from a haphazard sample of 100) that contain labelled mitochondria after each assay.

### *Competitive fertilisations*

Within each block, eggs from the 6-8 females were standardised to  $5.0 \times 10^4$  cells  $\text{mL}^{-1}$  and equal volumes from each female were mixed to form a common egg pool (Fig. 5.1). This egg pool was then divided into six separate 6 mL aliquots (one aliquot per petri dish) in preparation for the competitive fertilisation trials. Sperm from each of the six males within the block were standardised to  $5.0 \times 10^5$  sperm  $\text{mL}^{-1}$  and divided into six separate 950  $\mu\text{L}$  aliquots (i.e. 36 aliquots across the six males; Fig. 5.1). Separate subsamples of sperm were also retained for sperm trait analyses (described in detail below). In one of the six 950  $\mu\text{L}$  aliquots for each male, 50  $\mu\text{L}$  of 500 nM MitoTracker Green FM (Molecular Probes) mitochondrial dye solution was added (prepared as described in Chapter 2). In the other five aliquots we added 50  $\mu\text{L}$  of filtered seawater. We then performed six competitive fertilisation trials in which sperm from each of the six males were systematically dyed, combined with undyed sperm from the remaining five males, and added to the egg pool aliquot. Thus, across all six competitive fertilisation trials within each of the 20 blocks, we estimated each male's relative fertilisation success against all other males within the block (see Fig. 5.1). The fertilisation conditions imposed in our trials resulted in a sperm:egg ratio of 10:1 ( $5.0 \times 10^5$  sperm  $\text{mL}^{-1}$  in a total of 6 mL across the males and  $5.0 \times 10^4$  eggs  $\text{mL}^{-1}$  in 6 mL of egg pool), which avoids 0% or 100% fertilisation rates in this species (Oliver and Evans 2014; see also Chapter 2). Although fertilisation itself occurs rapidly upon addition of sperm, we waited 10 minutes to allow the mitochondria transferred from dyed sperm to become visible inside fertilised eggs (Chapter 2). We then counted the number of eggs containing labelled mitochondria from a haphazard sample of 100 for each of the six trials in a block (i.e. eggs fertilised by each of the six competing males).

### *Sperm traits*

We measured sperm motility traits for each male using computer-assisted sperm analysis (CASA; Hamilton-Thorne CEROS). Given recent arguments that sperm phenotypic traits should be assessed in the context of the female reproductive environment in which ejaculates compete (Fitzpatrick and Lüpold 2014), we measured sperm motility in solutions of 'egg water' separated from the common pool of eggs used in the fertilisation trials. Egg water, i.e. seawater in which the eggs had been spawned, contains chemical attractants that alter sperm phenotypes (Oliver and Evans 2014; Kekäläinen and Evans 2016). Such egg chemoattractants have important effects on the effective target size of eggs in broadcast spawners, on the scales mimicked by our fertilisation

trials (Jantzen et al. 2001; Riffell et al. 2004). Moreover, previous work has shown male-by-female interactions can be caused by egg water (Evans et al. 2012; Oliver and Evans 2014; Kekäläinen and Evans 2016). We control for these effects by using egg water from the common pool of eggs used for the competitive fertilisation trials. Thus, sperm responses are observed in an average female environment, which is likely to be more representative of the (multi-individual) spawning environment in which sperm would normally compete for fertilisations. To obtain egg water for each block, we filtered a subsample of the common egg pool through a mesh of 30  $\mu\text{m}$  pore size and collected the filtrate (Oliver and Evans 2014).

Sperm from each male in the block were standardised to  $2.0 \times 10^6$  cells  $\text{mL}^{-1}$ , and aliquots from each male were added to equal volumes of egg water (i.e. final concentration of  $1.0 \times 10^6$  sperm  $\text{mL}^{-1}$ ). This concentration was chosen to ensure sufficient motile sperm were present in the field of view for each CASA video, while remaining well within the maximum recommended concentration for CASA (Lu et al. 2014). We placed 5  $\mu\text{L}$  sperm solution (mixed with egg water) from each male onto separate wells of a 12-cell multi-test slide, which had been pre-washed in 1% polyvinyl alcohol to prevent sperm sticking to the surface. We then used CASA to record sperm motility parameters for each male's sperm (which are highly repeatable within males for *M. galloprovincialis*; Fitzpatrick et al. 2012), with threshold values for defining static cells of 19.9  $\mu\text{m}/\text{s}$  average path velocity and 4  $\mu\text{m}/\text{s}$  straight-line velocity. Many measures are provided by CASA essentially measure the same traits and are very strongly correlated; therefore, including all possible CASA measures in a multivariate selection analysis could result in multicollinearity problems (Lande and Arnold 1983). We chose motility parameters that measure different traits and thus were not expected to be perfectly correlated (see Results): flagellum beat cross frequency (BCF), curvilinear velocity (velocity along the sperm's swimming path; VCL), path linearity (LIN) and percentage of motile sperm (PM). Previous work has shown that these parameters predict fertilisation success under non-competitive conditions in *M. galloprovincialis* (Fitzpatrick et al. 2012; Oliver and Evans 2014).

Subsamples of sperm from each male were also preserved in 1% buffered formalin for subsequent measures of sperm length. To measure sperm length, we captured photographs of 30 individual sperm per male from the preserved samples using an EOS 600D digital camera mounted to an Olympus BX41 microscope at 800 $\times$  magnification. We then measured sperm head length and flagellum length from these photographs using ImageJ v1.48 (Abramoff et al. 2004; Collins 2007).

We did not record sperm midpiece length, as the sperm midpiece in *Mytilus* spp. is underdeveloped and not easily visible under light microscopy (Oyarzún et al. 2014). Within-sample morphology measures were similar across individual sperm cells (coefficients of variation for head and flagellum length were  $< 0.2$  for all samples). We calculated the mean head and flagellum length of the 30 sperm for each male.

### *Multivariate selection analyses*

We conducted multivariate selection analyses on the measured phenotypic traits in R version 3.3.2 (R Core Team 2017). These methods are based on the procedures outlined by Lande and Arnold (1983) to estimate linear ( $\beta_i$ ) and non-linear ( $\gamma_{ij}$ ) selection gradients from multiple regression of phenotypic traits on fitness. However, given our fitness measures (proportions) had non-normal residual distributions (which can affect standard errors and hypothesis tests of selection gradients; Morrissey and Goudie 2016), we used the modification described by Morrissey and Sakrejda (2013) for estimating selection gradients and their standard errors from general fitness functions. This method calculates linear and non-linear selection gradients as the first and second partial derivatives of absolute fitness with respect to multivariate phenotypic traits. First, we standardised sperm trait scores to a mean of zero and standard deviation of one (Lande and Arnold 1983). Second, we fit generalised linear models (GLMs) with logit link function to competitive fertilisation success using the R package 'mgcv' (Wood 2006). Third, linear and non-linear selection gradients were estimated from these models using the 'gsg' package of Morrissey and Sakrejda (2013). As correlation between linear and quadratic terms in regression based models can lead to incorrect estimates of  $\beta_i$  (Lande and Arnold 1983), we used a GLM with only linear terms when calculating  $\beta_i$  estimates and a GLM with all first and second-order terms when calculating  $\gamma_{ij}$  estimates. Note that because there was evidence of overdispersion for both GLMs (linear terms GLM dispersion parameter = 3.78, linear plus non-linear terms GLM dispersion parameter = 3.84), we used quasibinomial error distributions for these models. Standard errors and hypothesis tests of gradients were calculated using case bootstrapping (Morrissey and Sakrejda 2013). Our estimates for selection gradients using these methods were very similar to estimates obtained using the Lande and Arnold (1983) least-squares approach (Table S5.4).

We further investigated patterns of non-linear selection using canonical analysis (Phillips and Arnold 1989), which extends correlational selection analysis by identifying non-linear selection on axes that represent combinations of multiple traits (Blows and Brooks 2003; Blows 2007). We

performed a canonical rotation of the  $\mathbf{y}$  matrix (which contains quadratic selection gradients on the diagonal and pairwise correlational gradients on the off-diagonal) to identify the major axes of the response surface, eliminating the off-diagonal elements. These axes are represented by the eigenvectors of  $\mathbf{y}$ ,  $\mathbf{m}_i$ , with associated loadings of the original phenotypic traits. The eigenvalues ( $\lambda_i$ ) of  $\mathbf{y}$  are equivalent to quadratic selection gradients along the new axes defined by the eigenvectors. Linear selection gradients along the new axes ( $\theta_i$ ) can also be estimated by rotating the original  $\beta_i$  onto the new trait space (Phillips and Arnold 1989). Hypothesis tests of  $\lambda_i$  and  $\theta_i$  are usually conducted using the double regression approach, whereby the original trait scores are rotated onto the new trait space defined by the eigenvectors and a second-order regression is fit (Bisgaard and Ankenman 1996). However, this can lead to high type I error rates due to the false assumption that eigenvalues will be zero if no non-linear selection is present (Reynolds et al. 2010). To counter this problem, we used a permutation procedure for hypothesis tests of  $\lambda_i$  and  $\theta_i$ , which randomly shuffled fitness scores 1000 times, each time fitting second-order GLMs (again using quasibinomial errors to account for overdispersion; dispersion parameter = 3.84) of the rotated trait scores on permuted fitness and calculating linear and quadratic selection gradients in 'gsg' to generate null distributions of gradients. This method differs slightly from that of Reynolds et al. (2010), in that we did not perform a new canonical rotation for each permutation, as we were interested in selection along the particular axes defined by the eigenvectors of the original  $\mathbf{y}$  matrix (Lewis et al. 2011; Chenoweth et al. 2012; Ower et al. 2013).

We used non-parametric analyses to visualise fitness surfaces along significant axes of non-linear selection (on both the original and canonical scales). These surfaces represent visualisations of the geometric relationship between individual relative fitness (i.e. individual competitive fertilisation success divided by the population mean) and one or more trait axes (Phillips and Arnold 1989). Non-parametric approaches offer unconstrained visualisations when fitness surfaces take complex geometric forms (Schluter 1988; Schluter and Nychka 1994). Specifically, we used univariate cubic splines for surfaces along canonical trait axes (which are orthogonal to all other canonical axes), implemented in the R package 'mgcv'. Smoothing parameters were estimated to minimise generalised cross-validation (GCV) scores.

### *Phenotypic correlation analyses*

We calculated correlation coefficients and partial correlation coefficients between each pair of phenotypic traits. Partial correlation coefficients and p-values were calculated using the R package

'ppcor' (Kim 2015), with each partial correlation between pairs of traits calculated holding all other phenotypic traits constant.

## 5.4 Results

### *Multivariate selection analyses*

We found significant negative linear selection on flagellum length (Table 5.1), i.e. higher competitive success for males with relatively short flagella. There was no significant linear selection on any other phenotypic sperm trait (Table 5.1). The analysis of quadratic and correlational selection gradients revealed a significant positive correlational gradient on the cross product of VCL (sperm velocity) and flagellum length (Table 5.1), suggesting selection for a positive association between these two traits. There were no other significant quadratic or correlational selection gradients, although the negative correlational gradient on the cross product of LIN (sperm path linearity) and flagellum length was marginally non-significant at  $\alpha = 0.05$  ( $p = 0.058$ ; Table 5.1). Canonical rotation of the  $\boldsymbol{\gamma}$  matrix to further investigate non-linear selection revealed significant convex (i.e. negative quadratic) selection along the axis defined by eigenvector **m6**, which was associated with the largest negative  $\lambda$  (Table 5.2). This axis was primarily loaded positively by BCF (flagellar beat frequency) and VCL, and negatively by LIN and flagellum length (Table 5.2). There were no significant linear selection gradients ( $\theta_i$ ) along the new canonical axes (Table 5.2). Comparison of overall generalised linear model fits indicated that the models on the original trait axes (both linear only and linear plus non-linear gradients) explained relatively little variation in competitive success (Table 5.3). However, model fit was significantly improved when non-linear selection gradients on the canonical axes were specified (Table 5.3). This suggests that non-linear selection on the canonical axes was more important than linear or non-linear selection on the original traits. We therefore focus on the canonical axes of selection when interpreting these results.

The fitness surface for the canonical axis defined by **m6** indicated a peak for intermediate scores, with decreasing fitness for both strongly positive and strongly negative scores (Fig. 5.2). This suggests there is selection against combinations with (a) very high scores for BCF and VCL and very low scores for LIN and flagellum length (i.e. very fast sperm with highly curved swimming and short flagella), or (b) very low scores for BCF and VCL and very high scores for LIN and flagellum length (i.e. very slow sperm with very straight swimming and long flagellum).

**Table 5.1.** Linear selection gradients ( $\beta$ ) and the matrix of non-linear selection gradients ( $\gamma$ ) for the measured ejaculate traits.

	$\beta$	$\gamma$					
		BCF	LIN	VCL	PM	HL	FL
BCF	-0.071 ± 0.002	-0.145 ± 0.007					
LIN	0.032 ± 0.002	0.118 ± 0.005	-0.028 ± 0.006				
VCL	-0.006 ± 0.002	-0.018 ± 0.003	0.100 ± 0.003	-0.060 ± 0.004			
PM	-0.019 ± 0.001	-0.044 ± 0.004	-0.005 ± 0.004	0.010 ± 0.002	-0.049 ± 0.004		
HL	-0.052 ± 0.002	0.029 ± 0.006	-0.137 ± 0.005	0.052 ± 0.003	0.030 ± 0.004	0.061 ± 0.006	
FL	<b>-0.099 ± 0.001*</b>	0.032 ± 0.004	<b>-0.158 ± 0.004</b>	<b>0.144 ± 0.003*</b>	-0.022 ± 0.003	0.005 ± 0.002	0.037 ± 0.003

Note: BCF = flagellar beat frequency, LIN = path linearity, VCL = curvilinear velocity, PM = percentage of motile sperm, HL = head length and FL = flagellum length. For the non-linear selection gradients, quadratic gradients for individual traits are shown on the diagonal and correlational gradients for pairs of traits on the off-diagonal. All estimates are presented ± standard error. Significant selection gradients are shown in bold, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . The marginally non-significant correlation gradient between LIN and FL is also bolded.



**Table 5.2.** Estimates of linear ( $\theta$ ) and non-linear (quadratic,  $\lambda$ ) selection gradients on the new canonical axes (**m1-m6**) described by the eigenvectors of the  $\gamma$  matrix.

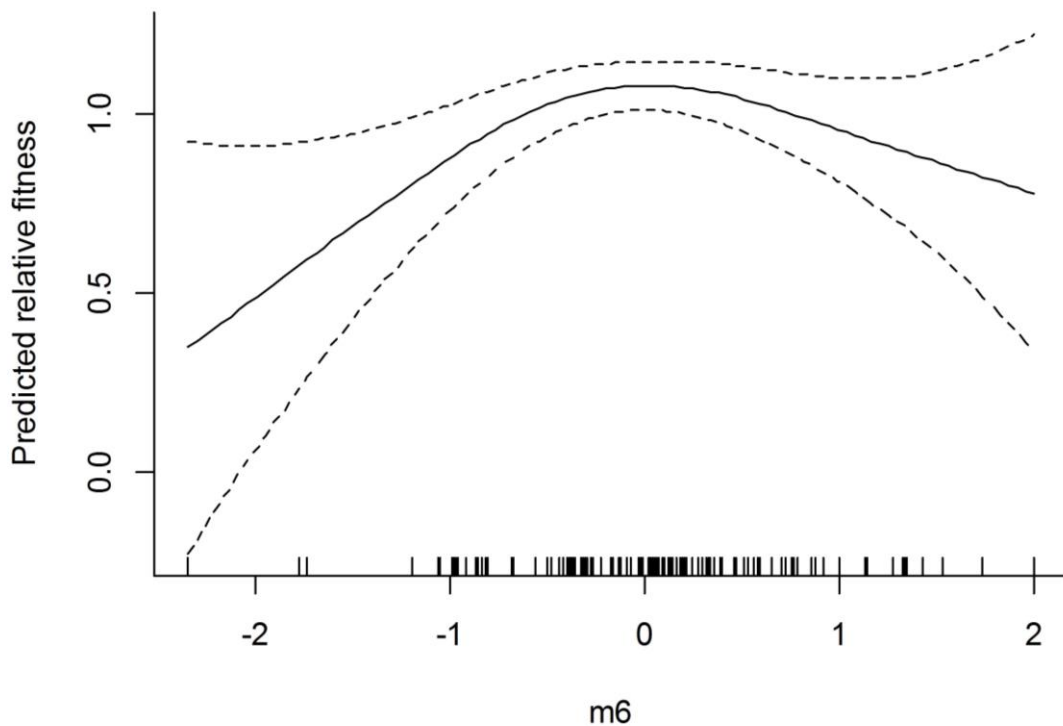
	$\theta$	$\lambda$	Trait loadings					
			BCF	LIN	VCL	PM	HL	FL
<b>m1</b>	-0.098 (0.082)	0.239 (0.090)	-0.109	-0.592	0.178	0.043	0.516	0.581
<b>m2</b>	0.038 (0.346)	0.100 (0.266)	-0.193	-0.346	-0.572	0.195	0.388	-0.572
<b>m3</b>	-0.020 (0.624)	0.026 (0.816)	0.256	0.427	0.441	0.221	0.665	-0.259
<b>m4</b>	-0.025 (0.584)	-0.033 (0.616)	0.427	-0.004	-0.229	-0.835	0.259	-0.022
<b>m5</b>	-0.083 (0.142)	-0.145 (0.128)	0.710	0.034	-0.434	0.461	-0.042	0.303
<b>m6</b>	0.002 (0.954)	<b>-0.370 (0.012)</b>	0.446	-0.588	0.454	0.034	-0.270	-0.419

Note: *P* values from permutation tests are shown in parentheses, with significant gradients bolded. Loadings of the original phenotypic ejaculate traits onto the canonical axes are also provided (BCF = flagellar beat frequency, LIN = path linearity, VCL = curvilinear velocity, PM = percentage of motile sperm, HL = head length and FL = flagellum length).

**Table 5.3.** Comparison of model fits for the generalised linear models containing (i) linear terms for the original traits, (ii) linear and nonlinear (quadratic and correlational) terms for the original traits, and (iii) linear and non-linear (quadratic) terms for the canonical axes.

Model	Residual deviance	Residual d.f.	$G^2$ (d.f.)	$P$
(i) Linear gradients	430.42	113	24.65 (6)	0.364
(ii) Linear + nonlinear gradients	352.02	92	103.05 (27)	0.473
(iii) Canonical axes gradients	352.66	107	102.42 (12)	0.002

Note: The overall significance of each model was estimated using likelihood ratio test, which calculate the test statistic ( $G^2$ ) as  $-2 \times$  difference in deviance between the model of interest and the null model and compare it to a  $\chi^2$  distribution (d.f. = difference in d.f. between the model of interest and the null model).



**Figure 5.2.** Individual relative fitness surface showing non-linear (convex) selection on canonical axis **m6**. Predictions for the surface were generated using a univariate cubic spline of **m6** scores on relative fitness (i.e. individual competitive fertilisation success divided by the mean across all males). The solid lines represent the fitted spline and the dotted lines represent  $\pm 1$  Bayesian standard error. Tick marks show the distribution of observed phenotypic scores along the **m6** axis.

#### *Phenotypic correlation analyses*

We detected several significant phenotypic correlations among the sperm traits (Table 5.4). There was a significant and moderately strong positive partial correlation (controlling for all other phenotypic traits) between BCF and LIN, significant but weak positive partial correlations between LIN and VCL and between VCL and PM, and a significant and moderate negative partial correlation between head length and flagellum length (Table 5.4). There was no evidence of multicollinearity among traits (variance inflation factor  $< 2$  for all traits; Table 5.4).

**Table 5.4.** Estimates of collinearity (variance inflation factor; V.I.F.) and matrix of phenotypic correlations among ejaculate traits.

	V.I.F.	Correlation coefficients				
		BCF	LIN	VCL	PM	HL
BCF	1.84					
LIN	1.79	<b>0.655 (0.623)***</b>				
VCL	1.10	-0.105 (-0.136)	<b>0.044 (0.184)*</b>			
PM	1.17	-0.332 (-0.172)	-0.309 (-0.150)	<b>0.244 (0.237)*</b>		
HL	1.17	-0.069 (-0.145)	0.043 (0.110)	-0.035 (-0.006)	-0.185 (-0.156)	
FL	1.15	0.099 (0.042)	0.082 (0.074)	0.047 (0.010)	0.127 (0.107)	<b>-0.337 (-0.313)***</b>

Note: The variance inflation factor estimates the increase in variance of parameter estimates due to collinearity among variables. BCF = flagellar beat frequency, LIN = path linearity, VCL = curvilinear velocity, PM = percentage of motile sperm, HL = head length and FL = flagellum length. Partial correlation coefficients, calculated with respect to all other measured traits, are shown in parentheses. Significant partial correlations are shown in bold \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

## 5.5 Discussion

Here, we demonstrate complex patterns of non-linear selection under sperm competition on combinations of sperm morphology and motility traits. Our experimental design represents an important advance for studies of post-ejaculatory sexual selection, by generating estimates of intrinsic male competitiveness that account for levels of variation in rival male and female genotypes found in natural populations (García-González 2008b; García-González and Evans 2011). In our externally-fertilising study system, we achieve this by simulating realistic conditions of multi-male and multi-female spawnings. For other systems, particularly internal fertilisers, males are likely to encounter different rival ejaculates and females sequentially across different matings. In such species, the principles we apply here could be extended to conduct multiple dyadic sperm competition trials with various rival males and females, and therefore obtain robust estimates of a male's sperm competition success relative to the population. Such estimates are important, as several previous studies have suggested that variation in ejaculate-by-ejaculate or ejaculate-by-female interactions among specific mating events could constrain the evolution of traits related to sperm competitiveness (e.g. Clark et al. 1999; Bjork et al. 2007). Our present results indicate that, despite the existence of genotype-by-genotype interactions at fertilisation in *M. galloprovincialis* (Evans et al. 2012; Oliver and Evans 2014), there are still overall patterns of selection on ejaculates across a biologically realistic range of competitive interactions. We conclude, therefore, that there is the potential for adaptive evolution of phenotypic sperm traits under sperm competition.

In our study, we measured selection on intrinsic sperm quality traits, specifically finding non-linear selection against divergent combinations of sperm velocity, swimming path linearity and flagellum length. Selection under sperm competition in external fertilisers is expected to act on such intrinsic traits, where sperm that are able to detect and reach eggs first in unpredictable external conditions will likely achieve relatively high competitive success (Evans and Sherman 2013). For internal fertilisers, with sperm storage and sperm displacement, reproductive success may depend less on intrinsic sperm traits and more on how ejaculates interact with rivals and the female reproductive tract at various post-mating stages (Pizzari and Parker 2009). For example, selection may favour sperm that are better able to move between female storage organs, remain viable during prolonged storage, or

modify their behaviour in response to rival ejaculates (e.g. Froman et al. 2002; Manier et al. 2010; Lüpold et al. 2012). In these systems, ejaculate traits themselves will likely need to be measured under competitive *in vivo* conditions to provide accurate patterns of selection. Regardless of the different traits expected to be important in external vs. internal fertilisers, our findings validate recent calls to view ejaculate traits as a functionally integrated target of selection (Pizzari and Parker 2009; Simmons and Fitzpatrick 2012; Fitzpatrick and Lüpold 2014); the selective patterns we report would be missed in a simple analysis of linear relationships between individual traits and competitive success.

We found convex selection across the observed range of phenotypes on canonical axis **m6** (loaded positively by BCF and VCL, and negatively by LIN and flagellum length). Selection favoured intermediate scores on this axis, when sperm were (a) slow with short flagella and curved swimming paths, or (b) fast with long flagella and straight swimming paths. This might reflect swimming strategies that allow sperm to efficiently track eggs. In external fertilisers, sperm locate eggs using local gradients of egg-derived chemoattractants (Jantzen et al. 2001; Riffell et al. 2004). When sperm are searching for such chemoattractants, slow and circular swimming is the most effective strategy (Friedrich and Jülicher 2008; Fitzpatrick et al. 2012), and once sperm have detected chemoattractants they swim in faster, straighter spirals toward eggs (Kaupp et al. 2006; Evans and Sherman 2013). In highly competitive environments, as experienced by ejaculates during synchronised spawning of mussel aggregations (Wilson and Hodgkin 1967; Villalba 1995; Gosselin 2004), faster and straighter movement along chemoattractant gradients is likely to provide an advantage over rivals (Yeates et al. 2013; see also Chapter 3). Since we measured sperm motility in the presence of chemoattractants, it is interesting that slow, circular swimming sperm still appear to have relatively high fitness. This unexpected finding may arise because we were unable to establish a true chemoattractant gradient when measuring sperm motility with CASA, which requires a relatively homogenised sample of egg water. Nevertheless, our results contribute to growing evidence that female-induced changes in ejaculate phenotypes can influence patterns of selection under sperm competition (Miller and Pitnick 2002; Lüpold et al. 2013, 2016).

We suggest two main areas for future research. First, we may gain a more complete understanding of how sexual selection targets ejaculates in external fertilisers by considering components other than sperm (e.g. Locatello et al. 2013; Bartlett et al. 2017), such as seminal fluid components (e.g. proteins or ions; Lahnsteiner et al. 2004; Rosengrave et al. 2009). Second, in order to predict evolutionary responses to multivariate selection, we need to understand the patterns of genetic variance and covariance underlying phenotypic traits (Lande 1979; Blows and Hoffmann 2005). Such analyses were beyond the scope of our current study, but studies in other species suggest that most ejaculate traits have high levels of additive genetic variation (reviewed in Simmons and Moore 2009), although genetic correlations among traits such as sperm morphology and motility may constrain evolution (Birkhead et al. 2005; Evans 2011; but see Gasparini et al. 2013). However, the location of genes underlying most ejaculate traits have yet to be established (Simmons and Moore 2009). We anticipate that investigation of the genetic architecture of ejaculate traits under selection could substantially improve our ability to predict evolutionary responses to sperm competition.

In conclusion, our investigation of multivariate sexual selection (i.e. selection under competitive conditions) on ejaculate traits reveals that selection acts on multiple sperm traits as an integrated phenotype, thus illustrating the importance of multivariate analyses for determining the effect of ejaculate traits in sperm competition. Our experimental design represents an important advance to the study of sexual selection on ejaculate traits, by providing biologically realistic estimates of intrinsic sperm competitiveness that are not confounded by post-zygotic effects and account for variation in rival male and female genotypes. Such estimates allow us to make inferences regarding the overall potential for adaptive evolution of ejaculate traits under sperm competition. We anticipate that such techniques, which provide robust estimates of intrinsic male competitiveness across reproductive contexts, will be helpful in elucidating the overall patterns of sexual selection on ejaculates in both external and internal fertilisers.

## **5.6 Acknowledgements**

We thank Cameron Duggin for help with mussel collections and experiments, Brighton Downing and Emma Daymond for experimental assistance, Michael Morrissey and Mark

Blows for helpful advice on multivariate selection analyses, and Alan Lymbery and two anonymous reviewers for helpful comments on the manuscript. Funding was provided by the UWA School of Animal Biology (RAL) and the Australian Research Council (JPE; Grant Number DP150103266). RAL was supported by the Hackett Postgraduate Research Scholarship and the Bruce and Betty Green Postgraduate Research Top-Up Scholarship.



## 5.7 Supplementary Material

### Supplementary Methods

#### *Simulations for number of males per block*

The aim of our experimental design was to obtain robust measures of competitive fertilisation success that accurately reflected a male's intrinsic competitiveness compared to the population of rivals. To achieve this, we first needed to determine the number of competing males per block required for a representative sample of the population variation in fertilising ability. Moreover, the number of competing males needed to be standardised across blocks, to ensure competitive fertilisation success (measured as a proportion) provided a comparable measure of fitness across males (i.e. offspring share from comparable reproductive events). Proportional data can present challenges as measures of fitness, as they measure the number of successes (the numerator) out of the number of attempts (the denominator). In some circumstances, this could lead to errors in the inference of selection (M. Morrissey, pers. comm.). For example, the relative number of offspring an individual contributes to the next generation might not necessarily reflect the proportion of successful attempts at reproduction if there are large discrepancies in the number of attempts per individual. Therefore, we needed to ensure the number of rival competitors and the opportunities for fertilisation were standardised across males to provide correct inference of selection.

In determining the optimum number of males per block, there is a trade-off between a sample size large enough to be representative of variation in male fertilising ability, and small enough to be logistically feasible in competitive trials (see main text for description of trials). To determine an appropriate sample size, we conducted simulations using data previously collected in our laboratory on non-competitive fertilisation success (using eggs from pooled females) of 119 *M. galloprovincialis* males (Fitzpatrick et al. 2012). We simulated block sizes from 2 - 10 individuals. For each block size, we used R version 3.3.2 (R Core Team 2017) to randomly sample the equivalent number of individuals from the data on 119 males, recorded the mean and variance of fertilisation success for the sample of males, and repeated the procedure 10,000 times. This generated a distribution of means and variances in fertilisation rates for each block size. We calculated the mean and 95%

confidence intervals (CIs) across the distribution of 10,000 means for each sample size, and compared these to the actual sample mean of all 119 males. For the variances, we calculated the median (as sample variances follow an approximately chi-square distribution that is bounded on the left by zero) and 95% CIs across the distribution of 10,000 variances for each sample size, and compared these to the actual sample variance of all 119 males. Note that the 95%CIs for the variances are not symmetric about the point estimate, given variance distributions are not symmetric.

As expected, the distributions of mean fertilisation success were all centred close to the overall mean, and the 95% CIs of the means narrowed as the block size increased from 2 – 10 (Table S5.1). There did not appear to be substantial improvements in the precision of the 95% CIs once block sizes were six or larger; at block sizes of six, 95% of the means were within approximately 6% of the overall mean fertilisation success, and increasing the sample size from 6 – 10 only improved this to within around 5% of the overall mean (Table S5.1). For the variance distributions, the median for blocks generally became closer to the overall variance in fertilisation success as group size increased, although the improvements in accuracy were not substantial beyond block sizes of five (Table S5.2). Similarly, for the 95% CIs of the variance distributions, the improvements in precision were not substantial at block sizes larger than five (Table S5.2). Therefore, we chose to use block sizes of six males in our competitive fertilisation trials, which the simulations suggested would provide a representative sample of fertilising ability across males while remaining logistically feasible.

#### *Assessing whether competitive fertilisation success was comparable across blocks*

Following our competitive trials (see main text), we estimated competitive fertilisation success as the proportion of fertilisations attributable to each male over all fertilised eggs in the block (i.e. controlling for any stochastic temporal variation in the readiness of eggs for fertilisation across blocks). To determine whether competitive fertilisation success varied systematically across blocks, we fit a binomial generalised linear mixed model with logit link function, with random effects of male ID nested within block. We assessed the significance of the random effects using likelihood ratio tests, which remove each effect from the model in turn and compare the fit of the reduced models against the full model ( $-2\times$  difference in log likelihoods compared against  $\chi^2$  distribution with 1 degree of freedom). We found

significant variation in competitive fertilisation success among males, indicating that males differed in their level of sperm competitiveness (Table S5.3). There was no significant variation in competitive fertilisation success among blocks, which indicated that competitive success measures were comparable across males from different blocks (Table S5.3).

### Supplementary Tables

**Table S5.1.** Results of simulated mean male fertilisation success for block sizes from 2-10, based on 10,000 iterations for each block size. The average and 95% confidence intervals (CIs) of the distribution of mean fertilisation success for each block size is shown, as well as the overall mean fertilisation success across all 119 males.

Simulated block size	Distribution mean	Lower 95% CI	Upper 95% CI
2	0.688	0.585	0.798
3	0.690	0.608	0.780
4	0.693	0.623	0.763
5	0.687	0.623	0.751
6	0.692	0.632	0.755
7	0.690	0.636	0.745
8	0.691	0.641	0.743
9	0.691	0.644	0.743
10	0.693	0.648	0.742
Overall mean	0.691		

**Table S5.2.** Simulated variances of male fertilisation success for block sizes from 2-10, based on 10,000 iterations for each block size. The median and 95% confidence intervals (CIs) of the distribution of variances for each block size is shown, as well as the overall variance in fertilisation success across all 119 males.

Simulated block size	Distribution median	Lower 95% CI	Upper 95% CI
2	$2.813 \times 10^{-3}$	$1.250 \times 10^{-5}$	$3.00 \times 10^{-2}$
3	$3.917 \times 10^{-3}$	$1.610 \times 10^{-4}$	$2.287 \times 10^{-2}$
4	$5.048 \times 10^{-3}$	$4.766 \times 10^{-4}$	$1.902 \times 10^{-2}$
5	$5.372 \times 10^{-3}$	$8.808 \times 10^{-4}$	$1.727 \times 10^{-2}$
6	$5.398 \times 10^{-3}$	$1.040 \times 10^{-3}$	$1.538 \times 10^{-2}$
7	$5.572 \times 10^{-3}$	$1.335 \times 10^{-3}$	$1.433 \times 10^{-2}$
8	$5.281 \times 10^{-3}$	$1.740 \times 10^{-3}$	$1.376 \times 10^{-2}$
9	$5.582 \times 10^{-3}$	$1.746 \times 10^{-3}$	$1.292 \times 10^{-2}$
10	$5.769 \times 10^{-3}$	$1.840 \times 10^{-3}$	$1.221 \times 10^{-2}$
Overall variance	$6.057 \times 10^{-3}$		

**Table S5.3.** Results of log-likelihood ratio tests for random effects of male ID and block on competitive fertilisation success. Full and reduced generalised linear mixed models were fit with binomial error distributions (logit link function). The likelihood ratio statistic ( $G^2$ ) for each random effect was calculated as  $-2 \times$  difference in log-likelihoods between the relevant reduced model and the full model. Probability (P) statistics were estimated by comparing  $G^2$  to a  $\chi^2$  distribution with one degree of freedom.

Model	Log likelihood	$G^2$	P
Full	-393.42		
(-Male)	-475.42	163.99	<0.001
(-Block)	-393.42	~0.00	~1.000

**Table S5.4.** Linear selection gradients ( $\beta$ ) and the matrix of non-linear selection gradients ( $\gamma$ ) for the measured ejaculate traits, calculated using the least-squares multiple regression technique of Lande and Arnold (1983). BCF = flagellar beat frequency, LIN = path linearity, VCL = curvilinear velocity, PM = percentage of motile sperm, HL = head length and FL = flagellum length. For the non-linear selection gradients, quadratic gradients for individual traits are shown on the diagonal and correlational gradients for pairs of traits on the off-diagonal. All estimates are presented  $\pm$  standard error. Significant selection gradients are shown in bold, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

	$\beta$	$\gamma$					
		BCF	LIN	VCL	PM	HL	FL
BCF	-0.091 $\pm$ 0.002	-0.066 $\pm$ 0.007					
LIN	0.061 $\pm$ 0.002	0.080 $\pm$ 0.005	0.021 $\pm$ 0.006				
VCL	-0.006 $\pm$ 0.001	-0.036 $\pm$ 0.003	0.092 $\pm$ 0.004	-0.105 $\pm$ 0.004			
PM	-0.040 $\pm$ 0.001	-0.069 $\pm$ 0.004	-0.033 $\pm$ 0.004	0.010 $\pm$ 0.002	-0.056 $\pm$ 0.005		
HL	-0.070 $\pm$ 0.002	0.020 $\pm$ 0.006	-0.106 $\pm$ 0.005	0.055 $\pm$ 0.003	0.032 $\pm$ 0.003	0.056 $\pm$ 0.005	
FL	<b>-0.11 <math>\pm</math></b> <b>0.002*</b>	0.071 $\pm$ 0.004	<b>-0.221 <math>\pm</math></b> <b>0.004*</b>	<b>0.166 <math>\pm</math></b> <b>0.002*</b>	0.006 $\pm$ 0.003	0.008 $\pm$ 0.003	0.021 $\pm$ 0.003



## **CHAPTER SIX**

### **General Discussion**





In this thesis, I undertook a detailed examination of the mechanisms and consequences of gamete-level sexual selection. As outlined below, this broad objective was addressed by applying a range of innovative experimental techniques that exploited the unique versatility of my study system, the broadcast-spawning mussel *Mytilus galloprovincialis*. Specifically, in the data chapters (Chapters 2-5), I: (a) developed a technique for measuring sperm competition outcomes at fertilisation; (b) demonstrated that differential attraction of sperm by egg-derived chemicals allows females to influence competitive fertilisation success; (c) explored molecular-level mechanisms (changes in gene expression) underlying the effects of egg chemoattractants on sperm; and (d) explored overall variation in sperm competitive success and patterns of multivariate sexual selection on ejaculates. Together, these approaches were designed to offer a holistic view of gamete-level interactions in this system, including both proximate mechanisms and adaptive outcomes.

### **6.1 Measuring competitive fertilisation success**

The method of using mitochondrial dyes in *M. galloprovincialis* to track the paternity outcomes of sperm competition (Chapter 2) allows quantification of competitive fertilisation success when controlling for post-zygotic factors such as differential embryo viability, thus overcoming a major conceptual hurdle faced in many sperm competition studies (García-González 2008a; García-González and Evans 2011). My work, therefore, complements advances made in other systems, particularly internally-fertilising model species, where transgenic lines that express fluorescent proteins in all cells have been used to quantify competitive fertilisation success (Manier et al. 2010; Droge-Young et al. 2012; Marie-Orleach et al. 2014). For example, in *Drosophila melanogaster* these lines have been used in studies that separate the effects of ejaculate-female and ejaculate-ejaculate interactions on sperm competitive success (Manier et al. 2010; Lüpold et al. 2012, 2013). However, in systems where the development and maintenance of breeding lines is not feasible, the measurement of competitive fertilisation success has remained challenging. The application of sperm dyes to ejaculates from any male (e.g. those sourced from natural populations), as applied here, could have important benefits for sperm competition studies.

The combination of (1) simple techniques for assigning competitive fertilisation success, and (2) the experimental versatility of externally-fertilising systems, provides considerable potential for elucidating the gamete interactions involved in post-ejaculatory sexual selection. Methods for labelling competing ejaculates, similar to those applied in Chapter 2 for *M. galloprovincialis*, could

be modified for use on other external fertilisers. Although paternal mitochondrial inheritance has not been described outside of bivalves, parts of the sperm that are universally transferred into fertilised eggs (e.g. DNA) have the potential to be utilised for labelling and tracking competitive success. For example, DNA fluorochromes have been used to track competitive success of ejaculates during *in vitro* fertilisations in mice (Martín-Coello et al. 2009; Firman and Simmons 2014, 2015), although *in vitro* fertilisations can be technically challenging in many internally fertilising systems. However, for many external fertilisers it is a logistically straightforward prospect to collect gametes from multiple individuals and perform complex split-clutch, split-ejaculate designs (as demonstrated in Chapters 3 and 5). Moreover, given recent arguments that post-ejaculatory sexual selection in external fertilisers has influenced the evolutionary history of sexual reproduction (e.g. Parker 2014), mechanistic investigations of competitive fertilisations in such systems could have taxonomically broad implications.

## **6.2 Sperm chemotaxis and gamete-level mate choice**

By applying the mitochondrial dye technique during competitive chemotaxis assays, I provided the first direct evidence that differential sperm chemotaxis can moderate intraspecific sperm competition (Chapter 3). This is an important advance in our understanding of the gamete-level mechanisms that drive competitive fertilisation biases, and raises the question of whether sperm chemotaxis might affect sperm competition outcomes in a broader array of taxa. Sperm chemotaxis is widespread across broadcast-spawning marine invertebrates (reviewed by Miller 1985; Eisenbach 1999) and mechanisms that enable females to differentiate among conspecific sperm are likely to be important in many of these species during synchronous spawning events (Levitan 2010; Evans and Sherman 2013). More broadly, several studies have demonstrated chemoattraction of sperm by female secretions (e.g. egg jelly or ovarian fluid) released with eggs in externally-fertilising vertebrates, including frogs (*Xenopus laevis*; Al-Anzi and Chandler 1998) and fishes (*Salmo salar* and *Salmo trutta*; Yeates et al. 2013), and there is tantalising evidence that such secretions can play a role in mediating competitive fertilisation success (Alonzo et al. 2016). Sperm chemotaxis also occurs in internal fertilisers (e.g. many mammalian species; Eisenbach and Giojalas 2006) and there are intriguing hints that it could affect sperm competition in such species. For example, Firman and Simmons (2015) found that eggs can bias paternity outcomes during competitive *in vitro* fertilisations in the house mouse (*Mus domesticus*), a species where egg-derived chemoattractants have previously been documented (Burnett et al. 2011). Similarly, in the internally fertilising guppy (*Poecilia reticulata*), Gasparini and Pilastro (2011) reported that ovarian

fluid (OF) has differential effects on sperm velocity that depend on the identity of sperm donors, and that OF ultimately biases paternity towards specific males. However, sperm chemotaxis (i.e. movement up a chemical gradient) was not identified as the proximate factor underlying gamete-level sexual selection in guppies, where the mechanisms behind OF-moderated gamete-level mate choice await further investigation. My results, along with this emerging body of work on other species, suggest that sperm chemoattraction may be a widespread (female-moderated) mechanism of influencing sperm competition across a broad range of taxa.

My findings add to the growing body of evidence that genetic compatibility effects might have widespread importance for female mediation of sperm competition (Birkhead and Pizzari 2002; Simmons 2005; Evans and Sherman 2013). For example, recent competitive fertilisation experiments using artificial inseminations in *P. reticulata* (Gasparini and Pilastro 2011) and *in vitro* fertilisations in *M. domesticus* (Firman and Simmons 2015), have shown that females can bias fertilisations toward sperm of unrelated (non-sibling) males. Genetic relatedness between males and females has also been found to influence competitive fertilisation success in Peron's tree frog (*Litoria peronii*; Sherman et al. 2008), although in this case sperm from genetically similar males were preferred (possibly to avoid the risk of hybridisation with a sympatric sister species).

In broadcast spawners, several studies have found that fertilisation success and offspring survival is higher in multi-male than single-male fertilisations; moreover, the magnitude of this benefit increases when there are greater differences in compatibility between each male and the female (Evans and Marshall 2005; Marshall and Evans 2005; Aguirre et al. 2016). In these systems, the genetic patterns underlying compatibility have rarely been investigated, although findings in sea urchins suggest that conditions of sperm competition may result in selection on females to reduce compatibility with males that carry common genotypes at gamete recognition loci, thus avoiding polyspermy (e.g. Levitan and Ferrell 2006). My results in Chapter 3 for *M. galloprovincialis* suggest that genetic patterns underlying compatibility-based gamete choice could be even more complicated than previously thought. In this case, gamete choice might be a trade-off between both selection for unrelated males (inbreeding avoidance), and avoidance of incompatibilities brought about by the mixing of divergent phylogenetic lineages. These findings underscore the complexity of gamete-level mate choice, and we have yet to fully grasp the adaptive genetic basis of these patterns.

### 6.3 Molecular mechanisms underlying changes in sperm phenotype in response to egg chemoattractants

In Chapter 4 I undertook a detailed investigation of the molecular mechanisms underlying phenotypic changes to ejaculates when sperm encounter egg chemoattractants, demonstrating that exposure of sperm to these chemical signals induces differential gene expression. Extension of this work is required to test whether such differential gene expression underlies male-by-female variation in chemotactic effects (Evans et al. 2012; Oliver and Evans 2014; Kekäläinen and Evans 2016), which could be achieved by comparing expression levels of candidate genes across factorial blocks of sperm-chemoattractant pairings (analogous to the block design of Chapter 3).

A subset of the differentially expressed genes from Chapter 4 were matched to functional annotations, and represent promising candidates for further investigation of male-by-female variation in sperm responses to chemoattractants. Many of the identified protein functions have the potential to be part of signalling pathways involved in sperm motility, chemotactic movement, the acrosome reaction or capacitation. These protein functions suggest egg-derived signals are more complex than previously thought; most studies of chemoattractants have focused on individual sperm-binding molecules that vary little across species or genera (Kaupp et al. 2006; Jagadeeshan et al. 2015). Interestingly, a recent study of sperm chemotaxis by follicular fluid in humans found that the strength of chemotactic signalling depends on a range of fluid components (Brown et al. 2017). The results of Chapter 4 suggest that exploring sperm gene expression could be a promising avenue of research for clarifying variation in sperm-egg signalling.

The finding of differential gene expression in mature, ejaculated sperm could also have broader implications for our understanding of how sperm function is controlled. Traditional dogma assumes that the structure and function of sperm cells are under diploid male control (Hecht 1998), which would avoid sperm-male conflicts and competition among haploid sperm genotypes within an ejaculate (Joseph and Kirkpatrick 2004; Immler 2008). However, there is growing evidence of transcription and haploid gene expression during post-meiotic spermatogenesis (e.g. Zheng et al. 2001; Vibrationovski et al. 2010). Moreover, sperm phenotypes can vary considerably within an ejaculate, and several studies have found that differences in phenotypes of fertilising sperm covary with offspring fitness (in the ascidian *Styela plicata*; Crean et al. 2012; salmon, *Salmo salar*; Immler et al. 2014; and zebrafish, *Danio rerio*; Alaviioon et al. 2017). Nevertheless, the possibility of post-ejaculation changes in gene expression has not yet been explored.

On the one hand, differential expression of sperm genes could afford plasticity in functional responses of sperm to external stimuli, as suggested by the results of Chapter 4. However, the changes in sperm gene expression that I report occur in response to ejaculate-female interactions, which raises the possibility that they are under female control (supported by the findings of Chapter 2 that differential chemotaxis allows females to bias competitive fertilisations). Additionally, sperm gene expression could also increase the potential for within-ejaculate phenotypic variation and conflicts between haploid and diploid interests (Hosken and Hodgson 2014). The exploration of post-ejaculation sperm gene expression, the mechanisms controlling it, and its adaptive consequences for males and females, could deliver many exciting avenues for future research.

#### **6.4 Sperm competitive success and post-ejaculatory sexual selection on ejaculates**

In my final experimental chapter, I returned to the question of overall variation in sperm competition success among males (found in Chapter 3), again applying the mitochondrial dye technique developed in Chapter 2. Despite the effects of male-by-female compatibility on competitive fertilisation success (Chapter 3), I found that variation in relative success among ejaculates from competing males led to patterns of post-ejaculatory sexual selection on sperm traits (Chapter 5). This is an important finding, as previous authors have suggested that male-by-male (i.e. ejaculate-by-ejaculate) and male-by-female (ejaculate-by-female) genotypic interactions on competitive fertilisations may limit selection on traits related to sperm competitiveness (Clark et al. 1999; Bjork et al. 2007). This highlights the power of experimental techniques that can (a) account for the potentially confounding effects of embryo viability on paternity estimates, and (b) estimate relative male reproductive success across a range of female and rival male genotypes. I anticipate that the application of such techniques to a wide variety of systems (see Chapter 5 Discussion) will have great utility for testing hypotheses about selection under sperm competition.

Understanding how selection under sperm competition targets ejaculate quality traits remains a key goal of post-ejaculatory sexual selection research (Snook 2005; Pizzari and Parker 2009; Fitzpatrick and Lüpold 2014). Although many empirical studies have identified particular ejaculate traits that predict competitive success, understanding how selection acts on ejaculates as a whole has proven challenging (Simmons and Fitzpatrick 2012). My findings from Chapter 5 emphasise the importance of adopting a multivariate approach for studying sexual selection on ejaculate traits;

this approach allowed me to detect complex non-linear selection against divergent combinations of sperm length, speed, and swimming path linearity. This complements and extends previous findings from non-competitive fertilisations in *M. galloprovincialis*, which also suggest non-linear correlational selection on multiple sperm traits (Fitzpatrick et al. 2012; see also Johnson et al. 2013 for context-dependent selection on multiple sperm morphology traits in an external fertiliser).

As discussed in Chapter 5, patterns of selection on ejaculate traits are expected to differ between external and internal fertilisers (see also Humphries et al. 2008; Simpson et al. 2014). For internal fertilisers, traits that allow sperm to negotiate storage and transport in the female reproductive tract, or directly interact with rival ejaculates, might be more important determinants of competitive success (Pizzari and Parker 2009). For example, a recent study of sexual selection on multiple ejaculate traits in *D. melanogaster* found that rival sperm influence each other's velocity, which likely affects the probability of successful retention in the female reproductive tract (Lüpold et al. 2012). By measuring multiple ejaculate traits under biologically realistic conditions expected in external and internal fertilisers, I envisage that our understanding of selection under sperm competition will be greatly enhanced.

## **6.5 Conclusion**

In conclusion, my thesis provides a detailed account of the mechanisms and adaptive outcomes of gamete-level sexual selection in a broadcast spawner. I anticipate that the experimental approaches developed and applied throughout my thesis may be adapted for a wide range of systems, with the potential to greatly enhance our understanding of the complexities of post-ejaculatory sexual selection. Moreover, my findings regarding (a) female mechanisms of gamete-level mate choice through sperm-egg chemical signalling, and (b) overall patterns of multivariate selection on ejaculates under sperm competition, address important unresolved questions in sperm competition research. Finally, I provide the first evidence of differential gene expression underlying post-ejaculation changes in sperm phenotypes, which is likely to have broad implications for our understanding of the way sperm function is controlled. I look forward to future research efforts that build on the patterns and processes identified throughout this thesis.

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**Appendix: PDFs of published chapters**

# SCIENTIFIC REPORTS



OPEN

## Fluorescent sperm offer a method for tracking the real-time success of ejaculates when they compete to fertilise eggs

Received: 07 December 2015

Accepted: 17 February 2016

Published: 04 March 2016

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Despite intensive research effort, many uncertainties remain in the field of gamete-level sexual selection, particularly in understanding how sperm from different males interact when competing for fertilisations. Here, we demonstrate the utility of broadcast spawning marine invertebrates for unravelling these mysteries, highlighting their mode of reproduction and, in some species, unusual patterns of mitochondrial inheritance. We present a method utilising both properties in the blue mussel, *Mytilus galloprovincialis*. In mytilids and many other bivalves, both sperm and egg mitochondria are inherited. We exploit this, using the vital mitochondrial dye MitoTracker, to track the success of sperm from individual males when they compete with those from rivals to fertilise eggs. We confirm that dying mitochondria has no adverse effects on *in vitro* measures of sperm motility (reflecting mitochondrial energetics) or sperm competitive fertilisation success. Therefore, we propose the technique as a powerful and logistically tractable tool for sperm competition studies. Importantly, our method allows the competitive fertilisation success of sperm from any male to be measured directly and disentangled from confounding effects of post-fertilisation embryo survival. Moreover, the mitochondrial dye has broader applications in taxa without paternal mitochondrial inheritance, for example by tracking the dynamics of competing ejaculates prior to fertilisation.

Darwin<sup>1</sup> first proposed sexual selection as an evolutionary force acting on variation in reproductive success caused by (1) intrasexual competition for mates (typically among males), and (2) intersexual mate choice (typically females choosing preferred males). Since then, sexual selection has become a major focus of evolutionary and behavioural research<sup>2</sup>. A critical turning point in the field of sexual selection was the recognition that females often mate with multiple males, or their eggs are exposed to sperm from multiple males, meaning that sexual selection can continue after gamete release<sup>3</sup>. This occurs as sperm competition, where ejaculates from rival males compete for fertilisations<sup>4</sup>, and cryptic female choice, where females influence the outcome of such contests<sup>5,6</sup>. Both of these mechanisms of sexual selection are widespread across most sexually reproducing taxa and constitute important evolutionary forces acting on both sexes<sup>7–9</sup>. Although these processes are commonly termed ‘post-copulatory sexual selection’<sup>10</sup>, we prefer the term ‘gamete-level sexual selection’ to include externally fertilising animals that do not pair or copulate.

Despite intensive research on gamete-level sexual selection, there remains a taxonomic bias toward mobile, terrestrial and internally fertilising animals<sup>3,11,12</sup>. In particular, relatively few studies have focused on broadcast spawning marine invertebrates. Typically, these animals have sedentary or sessile lifestyles and both sexes release gametes directly into the ocean, where fertilisation occurs<sup>13</sup>. Although largely neglected in the context of sexual selection (but see refs 11,14), broadcast spawners exhibit several attributes that make them ideally suited for understanding gamete-level sexual selection. First, the absence of mating competition or mate choice prior to gamete release means that sexual selection operates exclusively through gamete-level interactions<sup>11</sup>. Second, broadcast spawners offer highly tractable systems for controlled *in vitro* experiments on gamete-level interactions. This tractability has been utilised in recent studies to characterise patterns of multivariate selection on gametes<sup>15,16</sup>, examine variation in male–female gametic and genetic compatibilities<sup>17,18</sup>, and explore the transmission

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Trait (difference between scores of undyed and dyed samples)	PC1	PC2
VAP: average path velocity	0.94	0.28
VCL: curvilinear velocity	0.57	0.80
VSL: straight-line velocity	0.98	0.04
STR: straightness	0.50	-0.73
LIN: linearity	0.85	-0.52
ALH: amplitude of lateral head displacement	0.12	0.87
BCF: beat cross frequency*	-0.89	0.07
Eigenvalue	3.93	2.29
Cumulative per cent of variance explained	56.15	88.87

**Table 1. Sperm motility traits and principle components generated from the sets of differences in values between dyed and undyed sperm samples for each trait.** Shown are trait loadings, eigenvalues and cumulative per cent of variance in composite trait variables of the first two principle components. \*BCF was square root transformed prior to analyses.

of non-genetic paternal effects through sperm<sup>19–21</sup>. Finally, because broadcast spawning is likely the ancestral animal reproductive strategy<sup>22,23</sup>, the selective forces shaping this form of reproduction may yield insights into early evolutionary transitions, such as anisogamy to isogamy and external to internal fertilisation (for a recent theoretical model of this “sexual cascade” of events, see ref. 24).

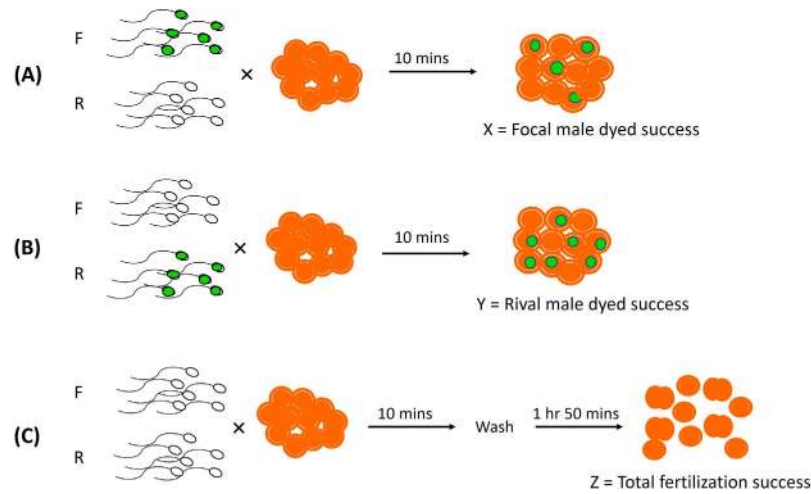
One key challenge facing researchers studying gamete-level sexual selection is to determine male reproductive success at the moment of conception. Even in external fertilisers, where gamete interactions are not hidden, there are considerable logistical challenges in identifying the outcome of sperm competition at fertilisation. Sperm competitiveness has typically been estimated through paternity analyses, which involves assigning offspring parentage among two or more putative sires using genetic markers<sup>8</sup>. Although paternity success is clearly an important component of a male’s reproductive fitness, its use in understanding sperm competition potentially confounds variation in embryo viability with variation in fertilisation success<sup>25–27</sup>. Embryo viability can be influenced by post-competition factors such as genetic sire effects<sup>28,29</sup>, genetic compatibilities between males and females<sup>18,30,31</sup>, maternal allocation<sup>32–34</sup> and non-genetic paternal effects<sup>20,21</sup>. Moreover, distinguishing between sperm competitive success on the one hand and offspring fitness on the other is crucial for evaluating whether processes such as ‘good sperm’ or ‘compatible genes’ underlie gamete-level sexual selection<sup>26</sup>.

Here, we propose a technique for directly examining competitive fertilisation success, using the broadcast spawning blue mussel, *Mytilus galloprovincialis* (Lamarck, 1819) as a model system. *Mytilus galloprovincialis* is a sessile, broadcast spawning bivalve with several characteristics that make it an ideal putative model system for gamete-level sexual selection. Individuals form large aggregations on intertidal substrates in temperate zones and both sexes spawn synchronously during winter months, meaning sperm and eggs from multiple individuals come into contact during each reproductive event. Moreover, unlike most animals, many bivalves (including *Mytilus* spp.) inherit mitochondrial DNA (mtDNA) from both parents in a phenomenon known as doubly uniparental inheritance (DUI)<sup>35</sup>. While the ultimate fate of paternal and maternal mtDNA differs depending on the sex of the offspring<sup>36</sup>, all embryos initially contain mitochondria transferred from the father’s sperm<sup>37,38</sup>. This presents the opportunity of labelling a male’s sperm with a vital fluorescent mitochondrial dye, allowing these sperm to compete with (undyed) sperm from other males, and tracking the real-time competitive fertilisation success of labelled sperm by counting eggs with labelled mitochondria. We develop the protocol for using this technique in evaluating sperm competition, using the mitochondria-specific vital dye MitoTracker Green FM. We test, in paired designs, whether the mitochondrial dye has any adverse effects on sperm motility and competitive fertilisation success.

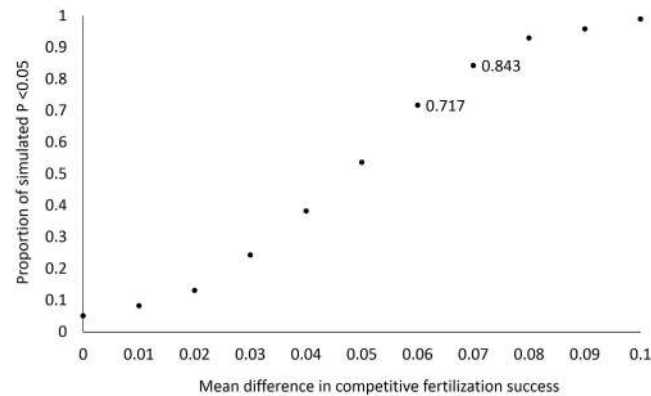
## Results

**Determining the effect of mitochondrial dye on *in vitro* measures of sperm motility.** We used computer-assisted sperm analyses (CASA) to evaluate sperm motility in two ejaculate samples from each of 18 males, one sample dyed with MitoTracker Green and the other left undyed. This paired experimental design therefore contrasted sperm motility between treatments while controlling for differences in ejaculate traits attributable to variation among males (see Methods). We first estimated the percentage of motile sperm from the total cell count for each sample, which was not significantly different between dyed and undyed samples (paired *t*-test,  $t_{17} = -0.62$ ,  $P = 0.54$ ). We estimated seven motility traits from the motile sperm: (1) average path velocity (VAP; velocity over smoothed sperm path); (2) straight-line velocity (VSL; average velocity on a straight line from start to end of path); (3) curvilinear velocity (VCL; average velocity on actual path); (4) straightness (STR; ratio of VSL to VCL); (5) linearity (LIN; ratio of VAP to VCL); (6) beat cross frequency (BCF; flagella beat rate); (7) amplitude of lateral head displacement (ALH; magnitude of sperm head displacement about the sperm trajectory). As sperm motility traits were highly correlated, we first calculated the difference in each trait between the dyed and undyed samples of each male, then reduced the set of differences in traits to principle components (PCs). Two PCs with eigenvalues >1 (collectively accounting for 88.87% of the variance in trait differences) were retained for the analysis. The first PC was loaded positively by the differences in VAP, VSL and LIN, and negatively by the difference in BCF, while the second PC was loaded positively by the differences in VCL and ALH and negatively by the difference in STR (Table 1). The means of the males’ scores for each PC (representing the composite difference between





**Figure 1. Experimental design for test of the effect of MitoTracker Green on competitive fertilisation success.** Each paired comparison involved three crosses between sperm from the same two competing males (focal and rival) and eggs from the same female. **(A)** Focal male's sperm dyed to estimate X; **(B)** rival male's sperm dyed to estimate Y; **(C)** both males' sperm undyed and overall fertilisation rate, Z, measured. The focal male's dyed success, X, was then compared to his undyed success, estimated as Z-Y.



**Figure 2. Proportion of significant simulated tests of varying differences in competitive fertilisation success between stained and unstained sperm (based on the variation and sample size in our dataset); i.e. power to detect a difference.** The horizontal axis shows the varying simulated differences in the probability of success due to staining, and the vertical axis shows the proportion of significant  $P$  values from 1000 simulated tests. A power of 0.8 falls between a difference of 0.06 and 0.07.

dyed and undyed measures for each male) were not significantly different from zero (PC1:  $t_{17} = 8.66 \times 10^{-16}$ ,  $P = 1$ ; PC2:  $t_{17} = 6.72 \times 10^{-16}$ ,  $P = 1$ ), thus confirming no discernible effect of the sperm dye technique on the *in vitro* measures of sperm motility.

**Competitive fertilisations.** To determine whether the MitoTracker dye had any effect on competitive fertilisation success, we conducted crosses with sperm from pairs of males (one termed the 'focal' male and the other one termed his 'rival') competing for the eggs of a single female (Fig. 1). For each cross (10 crosses,  $n = 20$  males and 10 females total) we performed reciprocal trials, where one male's sperm was dyed in each trial (i.e. focal dyed in trial A, rival dyed in trial B) to estimate the respective proportion of eggs he fertilised. We also determined the overall fertilisation rate in each cross (mean proportion of fertilised eggs  $0.733 \pm 0.050$  s.e.m., range 0.460–0.940). We then compared the dyed success of focal males to their success when undyed (estimated by the total fertilised eggs minus the rival male's success). There was no significant difference in the probability of successful competitive fertilisations between dyed and undyed samples (Wald  $t_{17} = 0.23$ ,  $P = 0.821$ ). As there were fewer replicate pairs than in the sperm motility trials, we conducted simulations to determine the power of our experimental design to detect differences in competitive fertilisation success between dyed and undyed sperm. These revealed we had 80% power to detect a difference in the proportion of fertilisation success of between 0.06 and 0.07 (Fig. 2). A mean difference of 0.06 was detected as significant in 72% of simulations, and a mean difference of 0.07 in 84% of simulations. These findings indicate that we had good power to detect small differences in fertilisation success

due to the dye treatment. Indeed, in our observed data the actual mean difference in fertilisation success between dyed and undyed samples was close to zero (mean difference  $-0.004 \pm 0.057$  s.e.m.; range  $-0.305$ – $0.310$ ).

## Discussion

Our method for visualizing the real-time success of sperm when they compete to fertilise eggs offers a tractable and potentially powerful tool for studying gamete-level sexual selection. Importantly, the MitoTracker Green mitochondrial dye had no detrimental effect on sperm behaviour and had no significant influence on the capacity of sperm to fertilise eggs when in competition with rival male ejaculates. Thus, the MitoTracker dye offers an effective and reliable method for visualising the outcome of sperm competition, with important benefits for research on gamete-level sexual selection. In particular, our proposed methods overcome a major hurdle in gamete-level sexual selection research, where success in sperm competition can typically only be inferred from offspring paternity assignment.

Our experimental confirmation that the Mitotracker dye had no discernible detrimental effects on patterns of sperm motility suggests that the dye does not disrupt sperm performance or mitochondrial function. Sperm motility traits have been linked to adenosine triphosphate (ATP) production in the sperm mitochondria<sup>39,40</sup>, or to the size of the sperm midpiece where mitochondria are located<sup>41</sup>. Furthermore, both sperm ATP content<sup>42</sup> and midpiece size<sup>43</sup> can vary with the level of sperm competition. Importantly, the motility traits we measured can have fitness implications for males during competitive and non-competitive fertilisations. For example, numerous studies have reported a positive association between sperm velocity and fertilisation success across a range of taxa (reviewed in ref. 8), although there are exceptions where slower sperm have been associated with greater fertilisation benefits (e.g. see refs 44,45). In *M. galloprovincialis*, males with slower sperm that swim in more pronounced curved paths are the most successful during non-competitive fertilisation trials<sup>15</sup>. This may reflect their capacity to search for eggs, or the chemical attractants released by eggs<sup>46</sup>, in a marine environment. Given our finding that the MitoTracker Green dye had no observable effect on these specific motility traits, we propose that it may be used to assess male reproductive fitness in future studies.

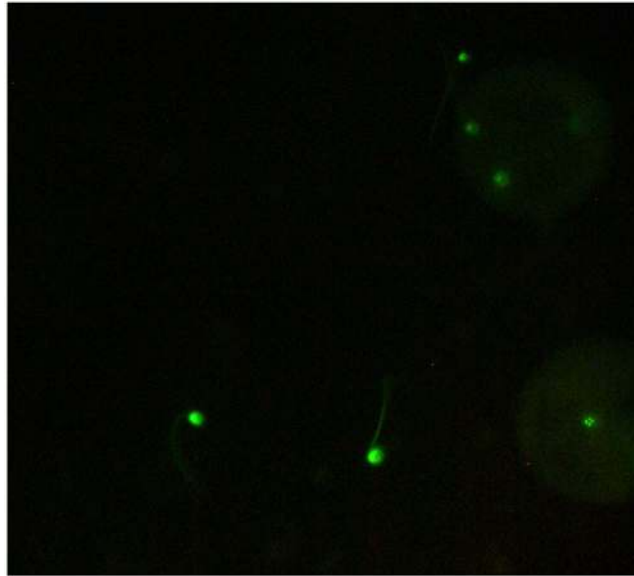
Consistent with the sperm motility results, we found that the mitochondrial dye did not significantly reduce competitive fertilisation success. Although there were fewer replicate pairs for this experiment than the sperm motility trials, our analyses had the power to detect small changes (proportional change of 0.06–0.07) in fertilisation success. This further suggests that the MitoTracker dye can be applied in a sperm competition context and provides a simple and cost-effective method for assessing competitive fertilisation success, negating the need to use offspring paternity assignment as a proxy for competitive fertilisation success. This is an important methodological advance because paternity success can be influenced by a range of factors operating after fertilisation, which may or may not be related to sperm competitive ability<sup>26</sup>. For example, in the sea urchin *Heliocidaris erythrogramma*, variance in embryo viability and fertilisation rates are uncorrelated within male-female pairings, suggesting that fertilisation rates cannot be inferred through variance in egg hatching rates<sup>17</sup>. In such systems, it is critical to use techniques that can directly estimate success at the point of fertilisation, such as the MitoTracker dye.

Previous studies using fluorescent dyes to distinguish competing sperm in fertility assays have mainly focused on domestic mammals. For example, dyes have been used to visualise the number of sperm from different males bound to bovine<sup>47,48</sup> and feline<sup>49</sup> eggs. However, these prior studies could not directly determine which of the bound sperm actually achieves fertilisation. By contrast, the present technique enables us to track the real-time success of individual sperm as they fertilise eggs. As such, our proposed method offers a potentially powerful tool in the context of understanding the dynamics of sperm competition. Other studies have overcome the challenge of identifying sperm from individual males through the use of selected genetic lines that express fluorescent protein, as for example in sperm of *Drosophila melanogaster*<sup>45,50,51</sup> and all cell types of *Macrostomum lignano*<sup>52</sup>. As with our study, these techniques make it possible to track the real-time success of sperm, although the logistical constraints of applying such methods to internal fertilisers mean that tracking sperm competition success *in vivo* is challenging in such systems. Moreover, the present technique does not require genetically modified lines for implementation, meaning that it can be applied to the sperm of any male, including those from natural populations.

The MitoTracker technique has broad applications not only across reproductive scenarios, but also potentially across taxonomic groups. The technique allows competitive fertilisation success to be tracked in any species with DUI, which include many species where knowledge about reproductive biology has potential commercial importance to fisheries<sup>36</sup>. Furthermore, we envisage that the mitochondrial dye technique has applications more broadly in taxa that do not have DUI of mtDNA. The mitochondrial dye could be used to track interactions of competing ejaculates *in vitro* by determining whether and how the presence of rival sperm influences pre-fertilisation performance, and examining if the capacity to influence rival sperm varies among males. For example, the use of selected lines expressing fluorescent proteins have revealed sperm displacement from the female reproductive tract by rival sperm in *M. lignano*<sup>52</sup>, and the adjustment of sperm swimming speed to match rival sperm in *D. melanogaster*<sup>45</sup>. We suggest that dyes such as MitoTracker could be used to explore such pre-fertilisation interactions in species where it is not possible to create selected genetic lines. To our knowledge, no studies have used dyes in this way to track interactions between competing sperm in an evolutionary context. We note that it may be necessary to test for the absence of an effect of dye on sperm performance in different taxa, although our expectation will be that the dye can reliably be used to stain sperm in other species. We look forward to the new insights that the implementation of this technique will bring to the field of sexual selection.

## Methods

**Sampling and spawning.** We collected mussels from Cockburn, Western Australia ( $32^{\circ}14'03.6''$ S,  $115^{\circ}76'25''$ E) during June to September 2014 and maintained them in aerated aquaria of recirculating seawater



**Figure 3.** *Mytilus galloprovincialis* sperm labelled with MitoTracker Green and fertilised eggs containing mitochondria from labelled sperm. Viewed using a Zeiss Axio Imager A1 fluorescent microscope, image captured using AxioCam MRC5 and Axiovision software. Image brightness and contrast adjusted using ImageJ software.

at the University of Western Australia until required for experiments (within 1–2 weeks of collection). Spawning was induced using a temperature increase from ambient to 28 °C<sup>15,53,54</sup>. Once an individual began spawning and its gender was determined, it was immediately removed from the water bath, washed in filtered seawater (FSW) to prevent contamination of gametes, placed in an individual 250 mL plastic cup and covered with FSW. Following spawning, egg densities were estimated by counting the number of eggs in a known volume under a dissecting microscope, and sperm densities were estimated from subsamples of sperm (fixed using 1% formalin) using an improved Neubauer haemocytometer. Gametes were then diluted to the concentrations required for trials (see below).

**Mitochondrial dye application.** We used the mitochondria-specific vital dye MitoTracker Green FM (Molecular Probes, Eugene, OR, USA) to stain sperm mitochondria (Fig. 3). We initially trialled a second dye colour (MitoTracker Red) but this proved to be unreliable in terms of consistency of uptake (no motile sperm were visibly labelled under fluorescence). Hence our competitive experiments involved reciprocally dyeing each competing male's sperm green, rather than labelling different males' sperm with different colours (see below). MitoTracker Green has been used previously to stain mitochondria of sperm in *Mytilus* spp. and other bivalves in order to follow paternal mitochondria through development<sup>38,55,56</sup>. We followed a protocol adapted from these studies for staining sperm in our experiments. All samples and solutions of dye were kept in the dark. Stock solutions of 1 mM dye were created by suspending 50 µg of MitoTracker Green in 74.5 µL dimethyl sulfoxide (DMSO). These were diluted with FSW to 10 µM working solutions. Sperm were stained in 1 mL samples (see below for sperm concentrations) containing 50 µL of working dye solution, i.e. 500 nM concentration of MitoTracker Green. Stained sperm were incubated in the dark for 10 minutes at room temperature. This was sufficient for uptake of dye by all cells in the sample (preliminary observations), while minimising sperm ageing effects, which are known to influence fertilisation rates in *M. galloprovincialis*<sup>15</sup>.

**Measuring sperm motility traits of dyed and undyed sperm samples.** Our first experiment compared the motility (swimming characteristics) of dyed and undyed sperm samples. We prepared two 950 µL subsamples of sperm at  $5 \times 10^6$  sperm mL<sup>-1</sup> from each of  $n = 18$  males; 50 µL of MitoTracker working solution was added to one subsample, and 50 µL of FSW to the other. After incubation of subsamples (see above), we placed 5 µL in a 12-cell multi-test slide, previously washed with 1% polyvinyl alcohol to avoid sperm sticking to the slide. Sperm motility was characterized using computer-assisted sperm analysis (CASA; Hamilton-Thorne CEROS, Beverly, MA, USA). We used threshold values for defining static cells of 19.9 µm/s VAP and 4 µm/s for VSL. For half the males, we measured undyed samples first, while in the other half, we measured dyed samples first. A mean of  $149 \pm 11.7$  s.e.m. motile sperm were recorded per sample. We calculated the percentage of motile sperm from the motile and total cell counts. We measured the following seven sperm motility parameters of the motile sperm, which are commonly used in studies of sperm competition and have high within-sample repeatability in *M. galloprovincialis*<sup>15</sup>: average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), straightness (STR), linearity (LIN), beat cross frequency (BCF), the amplitude of lateral head displacement (ALH).

**Competitive fertilisation trials.** To compare competitive fertilisation success of dyed and undyed sperm from the same males, we set up pairs of reciprocal competitive fertilisation trials in which sperm from the same

two males, one arbitrarily chosen as the ‘focal’ male and the other as his ‘rival’, competed for fertilisation of a single female’s eggs. In these trials, 1 mL samples of sperm from each male at concentrations of  $1 \times 10^5$  cells mL<sup>-1</sup> were added to 2 mL of eggs at  $1 \times 10^4$  cells mL<sup>-1</sup>; i.e. a final sperm:egg ratio of 10:1, shown in previous studies in this species<sup>54</sup> to avoid 0% fertilisation and ceiling effects (lower variation in fertilisation rates than expected<sup>15,57</sup>). In each fertilisation, only one male’s sperm was dyed; in (A), the focal male’s sperm was dyed, and in (B) the rival male’s sperm was dyed (Fig. 1). We then estimated competitive fertilisation success of the dyed sperm in each context as the proportion of eggs containing labelled mitochondria (e.g. see labelled eggs in Fig. 3). If we denote the focal male’s dyed success as  $X$  (from trial A) and his rival’s success as  $Y$  (from trial B), we expect  $X = 1 - Y$  if (i) the dye has no effect on sperm competition success, and (ii) in every trial all eggs were fertilised. We could not meet the second assumption, however, because raising sperm concentrations to levels that resulted in 100% fertilisation would have risked polyspermy, resulting in zygote failure<sup>58</sup>, and ceiling effects. We therefore conducted a third concurrent competitive cross (C), involving undyed sperm from the same two males to determine overall sperm fertilisation success,  $Z$ . With this estimate we set up a paired comparison in which, under a null hypothesis of no effect of dye, we expect  $X = Z - Y$  (Fig. 1). Ten paired comparisons ( $n = 20$  males, 10 females) were used in this experiment. For further detail regarding the fertilisation procedures, see the Supplementary Methods online.

**Data analyses.** Statistical analyses were carried out in R version 3.1.2<sup>59</sup>. For the sperm motility experiment, the percentage of motile sperm and all motility traits met the assumption of normality of differences between dyed and undyed values (Shapiro-Wilk tests,  $P > 0.05$ ), except BCF ( $W = 0.89$ ,  $P = 0.038$ ). Measures of BCF were therefore square root transformed before performing further analyses (after transformation:  $W = 0.91$ ,  $P = 0.083$ ). The percentage of motile sperm in dyed and undyed samples was compared using a paired  $t$ -test. To compare the sperm motility traits of males across dyed and undyed samples, we reduced the highly correlated traits to principal components (PCs) and used PC scores in  $t$ -tests. Specifically, we calculated the differences between trait scores of undyed and dyed sperm samples for each male and each trait, then performed a principal component analysis on the differences using the package ‘FactoMineR’<sup>60</sup> from which we retained PCs with eigenvalues  $> 1$ . The PC scores for males were used as sets of differences between undyed and dyed samples and tested with one-sample  $t$ -tests ( $H_0: \mu = 0$ ).

Dyed ( $X$ ) and undyed ( $Z - Y$ ) competitive fertilisation success estimates were also compared using paired analyses. Competitive fertilisation success, however, was a binomial response variable (i.e. proportions with denominator  $Z$ , the overall fertilisation rate). These data were modelled using a generalized linear mixed-effects model (GLMM) with a logit link function in the R package ‘lme4’<sup>61</sup>. The model was fit and parameters estimated using the Laplace approximation of the log-likelihood<sup>62</sup>. The model included the fixed effect of dye (i.e. dyed or undyed estimate) and a random effect for pair. The significance of the fixed effect was estimated using a Wald  $t$ -test, recommended by Bolker *et al.*<sup>63</sup> to account for uncertainty in overdispersion estimates, because our GLMM was overdispersed (residual deviance 53.18 on 17 degrees of freedom, dispersion parameter = 3.13). Overdispersion in mixed-effects models can also be accounted for by adding an observation-level random effect, with a separate level for each individual measurement<sup>64</sup>. In this case, adding an observation-level random effect resulted in underdispersion (residual deviance 2.83 on 17 degrees of freedom, dispersion parameter = 0.18), but did not change the conclusions regarding the fixed effect (Wald  $Z = -0.09$ ,  $P = 0.962$ , compare to test on original model in Results).

We conducted simulations to determine our power in the competitive fertilisation success experiment—i.e. the smallest difference in competitive fertilisation success between dyed and undyed samples that we could have detected with a power of 0.8 or more, given our sample size and the variation in our dataset. We provide a detailed procedure for the simulations in the Supplementary Methods online. Briefly, in each simulation we sampled 10 sets of paired values (dyed and undyed treatments) from binomial distributions in which a male’s sperm had a specified decrease in probability of fertilisation success when dyed. We then modelled these using a GLMM as described. We performed 1000 simulations for each specified difference in probability of success, and calculated the power of detecting that difference as the proportion of significant  $P$ -values out of 1000.

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## Acknowledgements

We thank Clelia Gasparini for help in developing the protocol for application of MitoTracker, Ashley Lymbery and Hannah Ashe for practical assistance, Jukka Kekäläinen and Cameron Duggen for assistance with collections, and Alan Lymbery and Samuel Lymbery for helpful comments on the manuscript. Funding was provided by the UWA School of Animal Biology (RAL) and the Australian Research Council (JPE). RAL was supported by the Hackett Postgraduate Research Scholarship and the Bruce and Betty Green Postgraduate Research Top-Up Scholarship.

## Author Contributions

R.A.L., W.J.K. and J.P.E. designed the study. R.A.L. conducted the experiments and analysed the data. R.A.L. wrote the first draft of the manuscript, and all authors contributed to the final version.

## Additional Information

**Supplementary information** accompanies this paper at <http://www.nature.com/srep>

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Lymbery, R. A. *et al.* Fluorescent sperm offer a method for tracking the real-time success of ejaculates when they compete to fertilise eggs. *Sci. Rep.* **6**, 22689; doi: 10.1038/srep22689 (2016).



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# Egg chemoattractants moderate intraspecific sperm competition

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Received August 3, 2017

Accepted November 10, 2017

Interactions among eggs and sperm are often assumed to generate intraspecific variation in reproductive fitness, but the specific gamete-level mechanisms underlying competitive fertilization success remain elusive in most species. Sperm chemotaxis—the attraction of sperm by egg-derived chemicals—is a ubiquitous form of gamete signaling, occurring throughout the animal and plant kingdoms. The chemical cues released by eggs are known to act at the interspecific level (e.g., facilitating species recognition), but recent studies have suggested that they could have roles at the intraspecific level by moderating sperm competition. Here, we exploit the experimental tractability of a broadcast spawning marine invertebrate to test this putative mechanism of gamete-level sexual selection. We use a fluorescently labeled mitochondrial dye in mussels to track the real-time success of sperm as they compete to fertilize eggs, and provide the first direct evidence in any species that competitive fertilization success is moderated by differential sperm chemotaxis. Furthermore, our data are consistent with the idea that egg chemoattractants selectively attract ejaculates from genetically compatible males, based on relationships inferred from both nuclear and mitochondrial genetic markers. These findings for a species that exhibits the ancestral reproductive strategy of broadcast spawning have important implications for the numerous species that also rely on egg chemoattractants to attract sperm, including humans, and have potentially important implications for our understanding of the evolutionary cascade of sexual selection.

**KEY WORDS:** Gamete interactions, genetic compatibility, sexual selection, sperm chemotaxis, sperm competition.

## Impact Summary

Gamete interactions are a critical component of competitive reproductive fitness. In many organisms, multiple mating (for internal fertilizers) or multi-individual spawning (for external fertilizers) lead to competition among ejaculates for fertilization and the opportunity for females (or eggs) to promote the success of preferred sperm. However, despite the pervasiveness of these forms of sexual selection, we know very little about the specific mechanisms of interaction among eggs and sperm that underlie such processes. One emerging putative mechanism is sperm chemotaxis, a taxonomically widespread phenomenon involving the attraction of sperm toward eggs by egg-derived chemicals. Here, we exploit the experimental versatility of a broadcast spawning mussel to provide the first empirical evidence that differential sperm chemotaxis allows females to bias the outcomes of intraspecific sperm competition toward sperm from “preferred” males. Additionally, patterns of

genetic relatedness at both nuclear and microsatellite markers suggest that females base these chemoattractant-induced preferences on complex patterns of genetic compatibility. Together, our results provide rare mechanistic insight into the interactions underlying gamete-level sexual selection. Moreover, this mechanism (sperm chemotaxis) has the potential to play similar roles across many taxa, given the ubiquity of egg chemoattractants. Indeed, as broadcast spawning was the ancestral mode of reproduction, gamete-level mechanisms that mediate competitive fertilizations likely played an important role in the evolution of sexual reproduction. The identification of such mechanisms, therefore, represents a crucial step forward in our understanding of sexual selection.

Sexual selection, which acts on variation in traits that influence reproductive success, almost certainly began in the sea with externally fertilizing organisms (Levitan 2010; Parker 2014). In

these systems, before the evolution of advanced mobility and sensory structures, there would have been limited opportunity for mating competition or mate choice prior to gamete release. Instead, synchronous broadcast spawning (where gametes from both sexes are expelled externally) and the co-occurrence of gametes from multiple individuals likely fuelled sexual selection in the form of sperm competition (competition for fertilization among ejaculates from multiple males; Parker 1970) and cryptic female choice (biasing of fertilization by females or their eggs toward particular ejaculates; Thornhill 1983; Eberhard 1996). Recent theory suggests that these ancestral processes of sexual selection instigated the evolutionary cascade toward many derived features of animal reproductive systems, including sexual dimorphism, internal fertilization, and precopulatory sexual selection (Parker 2014). However, sperm competition and cryptic female choice have themselves remained pervasive forms of sexual selection in most sexually reproducing taxa (Pitnick and Hosken 2010). There is, therefore, considerable empirical value in studying gamete-level interactions in extant broadcast spawners as they may provide clues into the mechanisms underlying sperm-egg interactions in a broad range of taxonomic groups (Levitan 2010; Evans and Sherman 2013).

A key goal in reproductive and evolutionary biology is to seek mechanistic insights into the processes that generate fertilization biases during sperm competition, and in particular into the role that females play in moderating this competition (Pitnick et al. 2009; Pitnick and Hosken 2010; Firman et al. 2017). While evidence for female control over fertilization is now compelling in many systems (e.g., Clark et al. 1999; Nilsson et al. 2003; Pilastro et al. 2004; Lovlie et al. 2013; Young et al. 2013; Firman and Simmons 2015), direct demonstrations of the underlying mechanisms remain largely elusive (but see Gasparini and Pilastro 2011; Alonzo et al. 2016). Broadcast spawning taxa offer particularly amenable and experimentally tractable systems with which to identify such mechanisms (Evans and Sherman 2013). Unlike internal fertilizers, in broadcast spawners the interactions between gametes are not hidden from view within the female reproductive tract, making it possible to visualize processes (e.g., gamete selection) that would otherwise have to be inferred indirectly. For example, eggs of broadcast spawners can moderate the recognition and fusion of sperm at the gamete surface (Palumbi 1999; Levitan and Ferrell 2006), or select specific sperm nuclei when multiple sperm penetrate the egg (Carré and Sardet 1984). However, eggs can also influence sperm remotely (i.e., prior to the meeting of gametes) through the release of chemical attractants. This process, which is known as sperm chemotaxis, is often crucial in broadcast spawners for ensuring eggs are found and fertilized by conspecific sperm (Miller et al. 1994; Riffell et al. 2004). Moreover, it has been argued that when ejaculates from multiple conspecific males are present, such remote signal-

ing between eggs and sperm could be an important mediator of competitive fertilization success (Evans et al. 2012).

Although sperm chemotaxis is taxonomically widespread in both external and internal fertilizers (Miller 1985; Eisenbach 1999; Eisenbach and Giojalas 2006), its putative role in gamete-level sexual selection has only recently come to light. For example, recent studies on the broadcast spawning mussel *Mytilus galloprovincialis* have revealed that chemoattractants have differential effects on the swimming behavior (chemotactic responses, swimming trajectory, and speed; Evans et al. 2012; Oliver and Evans 2014) and physiology (acrosome reaction; Kekäläinen and Evans 2016) of sperm from different conspecific males. The strength of these effects correlate with differences in offspring survival among male–female crosses (Oliver and Evans 2014). These findings suggest that chemoattractants could promote fertilizations by genetically compatible sperm, but this has yet to be investigated under conditions of sperm competition. Moreover, the molecular processes underlying potential genetic compatibility effects are unknown. For example, differential sperm chemotaxis may be driven by gamete-level mechanisms that promote optimal levels of general offspring heterozygosity, which is often cited as an explanation of compatibility-based gamete choice (Firman et al. 2017). Alternatively, more specific patterns of genetic compatibility may apply in *M. galloprovincialis* populations, which typically contain multiple mitochondrial DNA lineages as a result of historical migration patterns (Westfall and Gardner 2010; Dias et al. 2014). What is clear, however, is that the intraspecific effects of chemoattractants on fertilization have important fitness implications for both males and females in this system.

In this study, we test whether differential sperm chemotaxis moderates gamete-level mate choice in *M. galloprovincialis*, and whether fertilization biases attributable to differential chemotactic responses reflect underlying patterns of genetic complementarity. Our experimental design allows us to measure competitive fertilization success directly, rather than the more usual method of estimating fertilization success indirectly from a male's paternity share. The latter method (paternity share) can be confounded by postfertilization effects on offspring viability that may not be related to sperm competitiveness (García-González 2008a; García-González and Evans 2011). Here, we overcome this problem using a fluorescent dye to label the mitochondria of sperm of competing males (Lymbery et al. 2016). In *M. galloprovincialis* and many other bivalves, embryos inherit both paternal and maternal mitochondria through a process termed doubly uniparental inheritance (DUI) (Zouros et al. 1994; Obata et al. 2006; Breton et al. 2007). In DUI, maternal mitochondria are inherited in the somatic tissue of all offspring, while the paternal mitochondria are ultimately transmitted to the germ line of male offspring (Breton et al. 2007). Initially, however, sperm mitochondria are transferred into all fertilized eggs (Obata et al. 2006). This feature



of bivalve reproductive biology enables us to label sperm with a fluorescent mitochondrial vital dye and track their success during fertilization when labeled sperm from focal males compete with unlabeled rival ejaculates (Lymbery et al. 2016).

The primary aim of our study was to determine whether chemoattractants moderate competitive fertilization success in *M. galloprovincialis*. To test this we used a novel multistep experimental protocol involving multiple  $2 \times 2$  factorial crosses to determine whether egg chemoattractants moderate the success of ejaculates when they compete to fertilize eggs (see Methods). We also tested whether fertilization biases induced by egg chemoattractants (ECs) reflect patterns of genetic complementarity between focal sperm competitors and female EC donors. Our highly controlled design enabled us to: (1) directly examine variation in competitive fertilization success using sperm dyes, therefore controlling for postfertilization effects on embryo viability; (2) separate the effects of males, females, and their interactions on competitive fertilization success; and (3) isolate the effect of differential chemical attraction as the female-moderated mechanism for biasing competitive fertilizations. Importantly, our design controls for stochastic variation in fertilization that could be caused by random sampling of rival males, by using sperm from a standard rival to compete with the dyed sperm of focal males within each factorial (García-González 2008b; García-González and Evans 2011). Our ensuing results provide the first direct evidence in any system that differential attraction of sperm up an egg chemoattractant gradient moderates intraspecific competitive fertilization success. Furthermore, we find that fertilization biases induced by egg chemoattractants reflect both preferences for unrelated males at nuclear loci and the selection of the same mitochondrial DNA lineage, thus revealing the putative genetic benefits of gamete-level mate choice in this system.

## Methods

### STUDY SPECIES AND SPAWNING

*Mytilus galloprovincialis* is a sessile, gonochoristic bivalve mollusc that forms large aggregations on intertidal substrates in temperate regions of both Hemispheres. *Mytilus galloprovincialis* is distributed across the southern coast of Australia (Westfall and Gardner 2010), with phylogenetic studies indicating that populations contain signatures of both a native Southern Hemisphere lineage and a more recent introduction of Northern Hemisphere individuals (Westfall and Gardner 2010; Colgan and Middelfart 2011; Dias et al. 2014). Nevertheless, there appears to have been extensive reproductive mixture of individuals from these different lineages in Australian populations (Westfall and Gardner 2013). We collected mussels from Woodman Point, Cockburn, Western Australia ( $32^{\circ}14' 03.6''\text{S}$ ,  $115^{\circ}76' 25''\text{E}$ ) during the 2015 spawning season (June–September), and maintained them in aquaria of

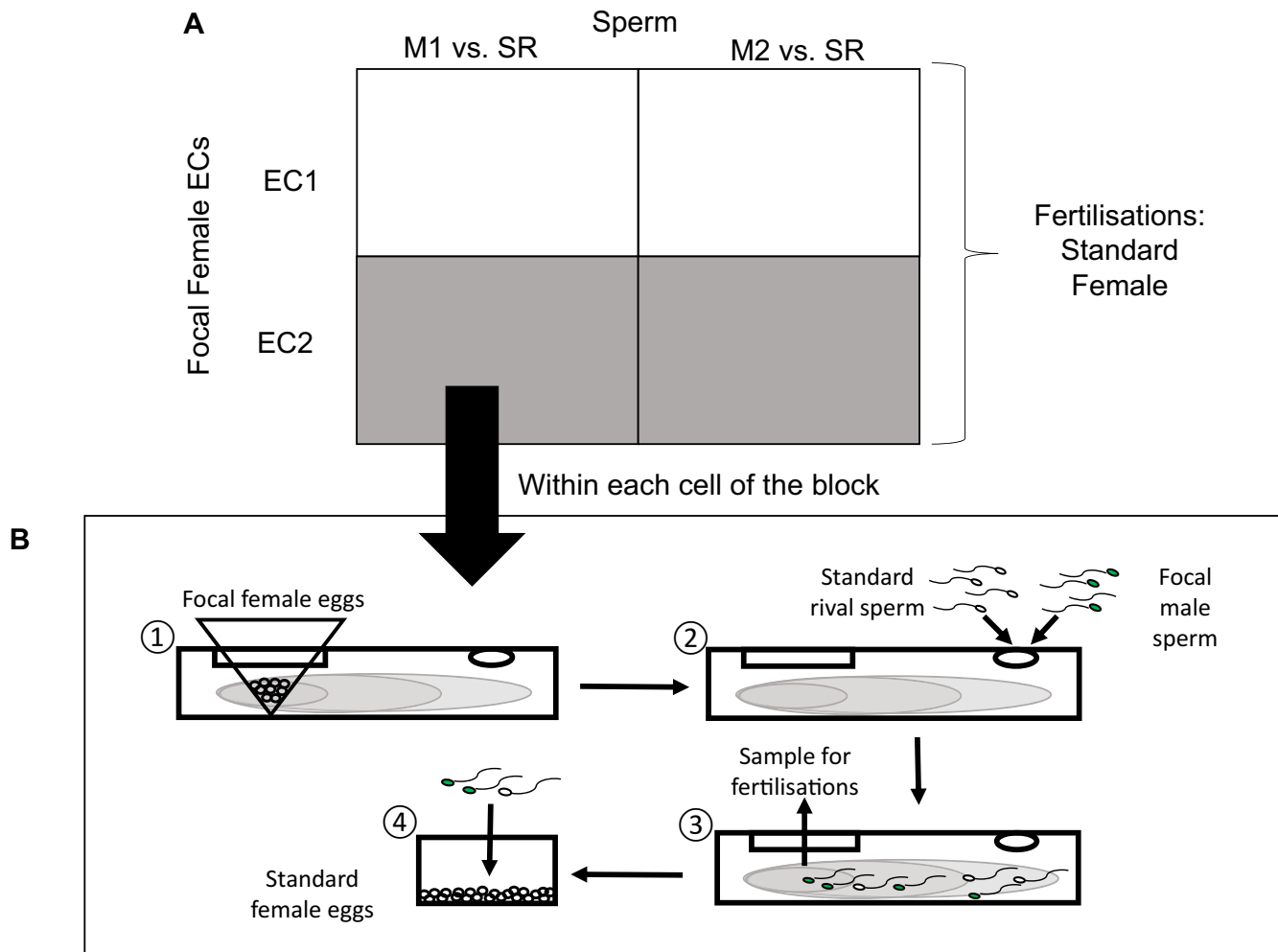
recirculating seawater at the University of Western Australia until required (within one week of collection). Spawning was induced using a temperature increase from ambient to  $28^{\circ}\text{C}$  (Lymbery et al. 2016). Once an individual began spawning and its sex was determined, we immediately removed it from the spawning tank, washed it in filtered seawater (FSW) to remove possible contaminating gametes, placed it in an individual 250 mL cup and covered it in FSW. Once gametes were suitably dense, we removed the spawning individuals, estimated egg concentration by counting the number of cells in a homogenized  $5 \mu\text{L}$  sample under a dissecting microscope, and estimated sperm concentration from subsamples (fixed in 1% formalin) using an improved *Neubauer haemocytometer*. We used these estimates to dilute gametes to their required concentrations for ensuing trials (see below).

### EXPERIMENTAL OVERVIEW

We used a multistep cross-classified design with blocks of two focal males (M1 and M2) and two focal females (F1 and F2) (Fig. 1A; the steps involved in a trial from a single cell of the block are shown in Fig. 1B). The initial steps involved differential sperm chemotaxis assays, where sperm from each focal male (dyed sperm, see below) competed with undyed sperm from a standard rival (SR) male in the presence of a chemoattractant gradient from each of the two focal females (EC1 and EC2). Therefore, four competitions were performed per block; M1 versus SR in EC1, M1 versus SR in EC2, M2 versus SR in EC1, and M2 versus SR in EC2. The final step involved competitive fertilization assays, where eggs from a single standard female (different to the focal females used for chemoattractant gradients) were used to assess the competitive fertilization success of the focal male (in competition with the standard rival) in each cross. This latter step enabled us to attribute differences in competitive fertilization success between competing ejaculates exclusively to the action of chemoattractant (i.e., it allows us to directly link differential chemotactic movement with the fitness outcome of sperm competition). Using eggs from a separate standard female for the fertilizations enables us to make this link by ensuring that within each block, the only source of male  $\times$  female variation in competitive fertilization rates is through differential chemoattraction. The standard female eggs, which were the same throughout all cells of the block, would have had no confounding effect on male  $\times$  female variation. We performed each competition in replicate, that is eight competitions per block (Fig. 1A), and conducted a total of 11 blocks (i.e.,  $n = 22$  focal males, 22 focal females, 44 male–female combinations, 88 competitions).

### COMPETITIVE CHEMOTAXIS AND FERTILIZATION TRIALS

In the first step of our experimental procedure, we established a chemoattractant gradient in an experimental chemotaxis chamber,



**Figure 1.** The overall design of an experimental block (A), and the steps performed within each cell of the block (B). (A) An example of one cross classified block, in which sperm from each of two focal males (M1 and M2) compete against sperm from a single standard rival (SR) in chemoattractant gradients from each of two focal females (F1 and F2). This generated four combinations per block, which were each replicated ( $n = 11$  blocks, 44 combinations, 88 competitions total). Eggs from a single standard female per block were used to estimate competitive fertilization success. (B) The multistep competition assay illustrated using a single combination from within a block. (1) Eggs from the focal female were suspended in filter mesh to generate a chemoattractant gradient within the chamber. (2) The mesh and eggs were removed after 1 h, and dyed sperm from the focal male and undyed sperm from the standard rival added to the other end of the chamber. (3) After 10 minutes, a subsample was taken from the center of the chemoattractant gradient. (4) The subsample was added directly to eggs from the standard female, and competitive fertilization success of the focal male was measured.

then allowed dyed focal (M1 or M2) sperm and undyed rival (SR) sperm to swim in the chamber (Fig. 1B; these steps were performed for each cell of Fig. 1A). The chambers were made from sterile syringes (Terumo), with the ends of each syringe sawn off and sealed with parafilm (Bemis) to form a 10 mL tube. A  $\sim 2$  cm<sup>2</sup> section was removed at one end of the chamber, and a small hole drilled in the other end. The chambers were fixed to a flat surface and a filter sack made of 30  $\mu$ m filter mesh was inserted through the square opening. We added 5 mL of FSW to the chamber and 2 mL of egg solution (at  $5 \times 10^4$  cells mL<sup>-1</sup>) to the filter sack, which retained eggs but allowed chemoattractants to disperse into the chamber. We left the chambers for 1 h to establish

a chemoattractant gradient (this time frame has previously been used to establish a chemoattractant gradient in larger chambers and we confirmed in preliminary trials that it was sufficient for our chambers; Evans et al. 2012).

Aliquots of sperm from the focal males and the standard rival were standardized to the same concentration (see below) and prepared for each competitive chemotaxis trial. The focal male's sperm was labeled using MitoTracker Green FM (Molecular Probes), prepared as described in Lymbery et al. (2016). In our previous study, we showed that dyeing sperm has no effect on sperm behavior or competitive ability (Lymbery et al. 2016). Apart from the addition of dye, focal male and standard rival sperm were

treated to the same procedure. Briefly, 950  $\mu\text{L}$  aliquots of sperm at  $1 \times 10^6$  cells  $\text{mL}^{-1}$  were prepared from each male, 50  $\mu\text{L}$  of 500 nm dye solution added to focal male aliquots, and 50  $\mu\text{L}$  of FSW added to rival aliquots. All samples (including undyed) were left in the dark (to prevent degradation of dye) for 10 minutes. The filter mesh containing focal female eggs was then removed from each chamber, and 500  $\mu\text{L}$  each of focal male and standard rival sperm solution added to the drilled hole at the opposite end of the chamber (Fig. 1B). Sperm were allowed to swim in the gradient for 10 minutes. Preliminary trials confirmed that this assay did not result in any contamination of nonfocal sperm by excess dye from focal sperm (see Supplementary Methods).

After focal and rival sperm had been in the chemotaxis chamber for 10 minutes, 1 mL samples were taken from the center of the chemoattractant gradient (see Fig. 1B) and added to a separate petri dish containing 1 mL of FSW with eggs from the standard female (diluted to  $1 \times 10^4$  cells  $\text{mL}^{-1}$ ). Prior to the addition of sperm, we rinsed the standard eggs with FSW through 30  $\mu\text{m}$  filter mesh to remove egg chemoattractants. However, even if these standard female eggs subsequently released chemoattractants, their impact (if any) would be to lessen our chance of detecting significant male-by-female effects (by obscuring patterns driven by the chemoattractants of focal females). Therefore, a significant male-by-female interaction in our analysis could only be attributable to the focal chemoattractants, which varied across the focal male samples. Moreover, fertilization occurs almost instantaneously upon the addition of sperm to the standard eggs (Lymbery et al. 2016), therefore decreasing the possibility that standard egg chemoattractants could reduce our power to detect effects. Although fertilization itself was instantaneous, we waited 10 minutes after the addition of sperm to allow dyed mitochondria to become visible inside fertilized eggs (Lymbery et al. 2016). We then estimated the fertilization success of the focal male under a fluorescent microscope by observing haphazard samples of 100 eggs, recording the numbers with and without dyed mitochondria.

Fertilizations from the rival (undyed) male were not scored, as estimating fertilizations from undyed sperm requires eggs to be left until they develop polar bodies, undergo cell division or until they can be assayed for survival. Therefore, the total numbers of fertilized eggs (dyed plus undyed) were not scored in this procedure. However, this is not required for the interpretation of the effects in our design, as we are not directly comparing the competitive success of focal males to rival males, but rather comparing the competitiveness of different focal males when they compete with a standard rival for standard eggs across different focal chemoattractants. Variation in the number of standard female eggs available for fertilization overall would only contribute to block-level variation (as all trials within a block used eggs from the same standard female) and therefore would not systematically change the relative share of paternity among focal males within

a block. Therefore, the male, female, and male  $\times$  female effects (all nested within block) on competitive fertilization were not confounded by variation in proportion of standard female eggs available for fertilization.

### NUCLEAR GENETIC RELATEDNESS

Foot tissue samples from all focal males and focal females (i.e., egg chemoattractant donors) were preserved in 100% ethanol. DNA was extracted using a salt-extraction method as described in Simmons et al. (2006) with the following alterations: tissue samples were incubated at 56°C overnight in the extraction buffer, and extracted DNA was resuspended in 100  $\mu\text{L}$  of sterile water. DNA concentrations were estimated using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and DNA samples were stored at  $-20^\circ\text{C}$  until required for PCR amplification. Each individual was genotyped at 13 polymorphic microsatellite loci; MGE002, MGE005, MGE008 (Yu and Li 2007), Mgu3 (Presa et al. 2002), Med744 (Lallias et al. 2009), MT282 (Gardestöm et al. 2008), MGES11 (Li et al. 2011), Mg-USC20, Mg-USC22, Mg-USC25, Mg-USC28, Mg-USC42, and Mg-USC43 (Pardo et al. 2011) (primer sequences provided in Table S1). Singleplex PCR reactions were run for each sample at each locus with a reaction volume of 5  $\mu\text{L}$ , containing 1  $\mu\text{L}$  MyTaq reaction buffer (Bioline), 0.2  $\mu\text{L}$  primer mix (solution containing 10 nM each of forward and reverse primer, forward primer fluorescently labeled), 0.5  $\mu\text{L}$  bovine serum albumin (Fisher Biotec), 0.1  $\mu\text{L}$  MyTaq DNA Polymerase (Bioline), 2.2  $\mu\text{L}$  sterile water, and 1  $\mu\text{L}$  DNA sample (approximately 10 ng). PCRs were performed using an Eppendorf Mastercycler epGradient S, with an initial denaturation step at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 54°C (MGE005 and MGE008) or 60°C (all other loci) for 1 min and 72°C for 1 min, with a final extension step of 72°C for 5 min. The PCR products were analyzed on an ABI 3730 96 capillary machine using a Genescan-500 LIZ internal size standard, and genotypes for each locus were scored using GENEMARKER software (SoftGenetics). Peaks identified by GENEMARKER were checked manually and adjusted as necessary to minimize scoring errors.

One locus (MGES11) was monomorphic for our samples, with the number of alleles for the other 12 loci ranging from 3–20. We examined patterns of subpopulation variation and clustering of nuclear genotypes using the software program STRUCTURE (Pritchard et al. 2000, 2007; Falush et al. 2003; Supplementary Methods). Pairs of loci were tested for genetic linkage using likelihood ratio tests in GENEPOP (Raymond and Rousset 1995; Rousset 2008), with one pair of loci in significant linkage disequilibrium (Med744 and Mg-USC22,  $P < 0.001$ ). We therefore removed one of these loci from the analysis, specifically Med744 as there was also evidence of null alleles at this locus (Table S2; null alleles estimated using MICROCHECKER software; Van Oosterhout

et al. 2004). There were excess homozygotes and evidence for null alleles at seven other loci (Table S2). However, removing all loci with null alleles can considerably reduce the power to detect variation in genetic relatedness and result in less accurate relatedness estimates than when all loci are included (Supplementary Methods; see also Robinson et al. 2013). We therefore used a maximum likelihood estimator that can account for null alleles (Kalinowski et al. 2006) to calculate genetic relatedness from the remaining 11 loci between each focal male–female pair in each block. These estimates were calculated using the ML-RELATE software package (Kalinowski et al. 2006). We compared these estimates to a range of other relatedness estimators and found consistent patterns of variation in relatedness across different methods, increasing our confidence in the reported measures of nuclear genetic relatedness (see Supplementary Methods). Moreover, to determine whether any markers had a disproportionate effect on measures of relatedness, we examined whether relatedness changed when each marker was removed in turn, and found little variation across different combinations (Table S3).

### MITOCHONDRIAL HAPLOTYPES

We sequenced female-type (F-type) CO1 mtDNA, which is generally considered to have a more reliable phylogenetic signal than male-type mtDNA and has multiple phylogenetic lineages in Australian *M. galloprovincialis* populations (Gérard et al. 2008; Colgan and Middelfart 2011; Dias et al. 2014). Using the DNA extracted as previously described, we amplified F-type CO1 haplotypes using PCR reagents and conditions as described in Dias et al. (2014). Samples were sequenced in both directions by the Australian Genome Research Facility, Perth. Consensus sequences were aligned, analyzed and trimmed in Geneious v 6.1.8 (Kearse et al. 2012) using the Geneious alignment feature with default parameters. A preliminary Neighbor-Joining tree was constructed from the 44 individuals to identify the number of unique sequences present ( $n = 14$ ; Table S4). We added 105 northern and southern *Mytilus* haplotypes of the COI gene to our unique sequence set, as compiled in Dias et al. (2014). We inferred phylogenetic relationships using MRBAYES V3.1.2 (Huelsenbeck and Ronquist 2001) in Geneious v 6.1.8. We set the parameters and performed the Bayesian analyses as described in Dias et al. (2014), with the modification that we used a GTR+G substitution model. We determined phylogenetic relationships from 75% majority-rule consensus of postburn-in trees.

### STATISTICAL ANALYSES

Analyses were performed using R version 3.3.2 (R Core Team 2016). We first analyzed competitive fertilization success of focal sperm as a binomial response variable (proportion of eggs successfully fertilized by dyed sperm in competition). We fit a GLMM with logit link function in the “lme4” package (Bates et al.

2014), using the Laplace approximation of the log-likelihood to estimate model parameters (Raudenbush et al. 2000). Our model included a fixed intercept term and random effects of male (overall variation among sperm of focal males), female (overall variation among focal female chemoattractants), male-by-female interaction (variation among sperm-chemoattractant combinations), and experimental block. There was no overdispersion in our model (residual deviance = 77.15 on 83 degrees of freedom, dispersion parameter = 0.93), and the scaled residuals (calculated using the “DHARMA” package; Hartig 2017) were uniformly distributed (Kolmogorov–Smirnov test;  $D = 0.053$ ,  $P = 0.967$ ). Focal male competitive fertilization success ranged from 0% to 44%, that is significantly lower than 50% (fixed intercept term of GLMM =  $-1.79$  [95% CIs =  $-2.11$ ,  $-1.47$ ], Wald  $Z = -1.78$ ,  $P < 0.001$ ). This was expected given only the subset of sperm that successfully traveled to the center of the chemoattractant gradient was used for fertilizations. We assessed the significance of random effect terms by removing each from the model in turn and compared the fit of the reduced models against the full model with likelihood ratio tests ( $-2 \times$  difference in log likelihoods compared against  $\chi^2$  distribution with 1 degree of freedom).

Next, we examined whether nuclear genetic relatedness and mitochondrial lineages of focal male and focal (i.e., chemoattractant-producing) female pairs were predictive of competitive fertilization success. The replicate measures of competitive fertilization success for each combination of focal sperm and focal chemoattractant were significantly repeatable ( $R = 0.044$  [95% CIs 0.023, 0.069],  $P < 0.001$ ; estimated using GLMM method in the “rptR” package; Nakagawa and Schielzeth 2010). Therefore, the replicate measures were combined into weighted means (i.e., total fertilized out of total number of eggs across the two replicates). We fit a GLMM with logit link function to competitive fertilization success, with a continuous fixed effect of nuclear relatedness and a fixed categorical factor specifying whether the focal male and focal female pair had the same mitochondrial lineage or a different lineage. We also fit random effects of male, female, and block. There was no evidence of overdispersion in our model (residual deviance = 11.91 on 37 degrees of freedom, dispersion parameter = 0.32), nor heteroscedasticity of scaled residuals (Kolmogorov–Smirnov test;  $D = 0.079$ ,  $P = 0.944$ ). We used Wald Chi-square tests to assess the significance of the fixed effects.

## Results

### COMPETITIVE FERTILIZATION SUCCESS

There were two sources of significant variation in focal male competitive fertilization success: (a) the male effect, and (b) the male-by-female interaction (Table 1). Although significant interactions often dictate that other effects must be interpreted cautiously,

**Table 1.** Results of log-likelihood ratio tests for random effects on focal male competitive fertilization success.

Model	Log likelihood	AICc	G <sup>2</sup>	P
Full	-282.94	576.60		
(-Male)	-285.89	580.26	5.90	0.015*
(-Female)	-283.27	575.01	0.66	0.417
(-Male × Female)	-285.41	579.30	4.95	0.026*
(-Block)	-283.84	576.17	1.81	0.178

Full generalized linear-mixed effects model included the proportion of eggs successfully fertilized by the focal male as the response variable (with logit link function), with random effects of focal male ID, focal female ID, male-by-female interaction and experimental block. The fixed intercept of the full model was significantly negative (intercept = -1.79 [95% CIs = -2.11, -1.47], Wald Z = -1.78,  $P < 0.001$ ). Estimated variance components associated with random effects are provided in Table S5. Reduced models were fit by excluding each random effect in turn. Akaike information criteria with correction for finite sample sizes (AICc) are provided for full and reduced models. The likelihood ratio statistic (G<sup>2</sup>) for each random effect was calculated as  $-2 \times$  difference in log-likelihoods between the relevant reduced model and the full model. Probability ( $P$ ) statistics were estimated by comparing G<sup>2</sup> to a  $\chi^2$  distribution with one degree of freedom.

in this case the removal of both the male effect and the male-by-female interaction resulted in a significantly worse fit than removal of the male-by-female interaction alone (likelihood ratio statistic  $G^2 = 68.80$ ,  $P < 0.001$ ). Therefore, the significant male effect suggests that there was variation among males in their average competitive success (i.e., some males were intrinsically “better” sperm competitors than others). The male-by-female interaction, on the other hand, indicates that there was significant variation in the way chemoattractants of focal females affected the competitive success of different focal males. In other words, the success of each focal male within a block depended on the specific identity of the focal female chemoattractant.

### GENETIC RELATIONSHIPS

The nuclear data indicated a well-mixed population (Fig. S1), despite F-type CO1 mtDNA haplotypes revealing signatures of two historical phylogenetic lineages (consistent with previously identified Northern and Southern Hemisphere lineages; Fig. S2; see also Dias et al. 2014). Nuclear genetic relatedness did not differ between focal male–female pairs that had the same mitochondrial lineage and those that had different mitochondrial lineages (two-sample  $t$ -test,  $t_{42} = 0.31$ ,  $P = 0.759$ ). We tested whether overall nuclear genetic relatedness or phylogenetic mtDNA lineages of focal male and focal (i.e., chemoattractant-producing) female pairs predicted patterns of gamete-level sexual selection (i.e., competitive fertilization success). We found significant main effects of both nuclear relatedness and mitochondrial lineage (Table 2). Specifically, competitive fertilization success was

**Table 2.** Effects of nuclear genetic relatedness and phylogenetic mitochondrial lineage on competitive fertilization success.

Fixed effect	Estimate	X <sup>2</sup>	P
Nuclear relatedness	-0.35 [-1.32, -0.02]	3.92	0.047
Mitochondrial lineage	0.35 [0.22, 0.65]	15.52	<0.001

Effects estimated from generalized linear-mixed effects models of the proportion of eggs successfully fertilized by the focal male (with logit link function), with fixed effects of nuclear relatedness and mitochondrial lineage and random effects of focal male ID, focal female ID, and experimental block. The final model did not include the interaction term of the fixed effects, as the interaction was nonsignificant in the full model (Wald  $\chi^2 = 0.93$ ,  $P = 0.335$ ) and its inclusion reduced model fit (see Table S6; although significance of the main effects did not change with inclusion of the interaction). The fixed intercept of the model was significantly negative (intercept = -1.58 [95% CIs = -1.95, -1.22], Wald Z = -9.08,  $P < 0.001$ ). Nuclear relatedness of focal male and focal female pairs was estimated from microsatellite loci using maximum likelihood (higher values = more closely related). Mitochondrial lineage (Northern or Southern Hemisphere) was assigned based on female-type CO1 sequences, with focal male and focal female pairs scored as belonging to different or same lineage (estimate represents the mean change in fertilization success on the latent scale from different to same lineage). Hypothesis tests of main effects were conducted using Wald  $\chi^2$  tests (d.f. = 1 for each effect).

higher when focal male and focal female nuclear genotypes were less related, but also when focal males and focal females had the same mitochondrial lineage.

### Discussion

Our results reveal that differential attraction of sperm up a chemical gradient can act as a mechanism of gamete-level mate choice. To our knowledge, this is the first direct evidence that egg chemoattractants influence intraspecific sperm competition, supporting the previously documented differential effects of egg chemoattractants on sperm swimming direction (Evans et al. 2012), sperm motility (Oliver and Evans 2014), and sperm physiology (Kekäläinen and Evans 2016). We show that the effect of chemoattractants on competitive fertilization success depends upon the particular combination of focal male and focal female, specifically favoring certain genetic combinations over others. Previous work on this system has shown that the strength of sperm chemotactic responses for any given male–female pairing is positively correlated with offspring survival (Oliver and Evans 2014). These previous findings, together with the present results, suggest that egg chemoattractants allow females to promote fertilization by more compatible males when multiple ejaculates compete. This provides rare insight into the mechanisms used by females to gain control over the outcome of sperm competition.

Our results complement and extend recent evidence that female reproductive fluids more broadly can have important roles in gamete-level sexual selection. In particular, there has been considerable interest in the ovarian fluid (OF) produced by various female fishes. In externally fertilizing salmonids, for example, OF released with eggs can differentially mediate the swimming speed of conspecific sperm depending on the particular male–female pairing (Urbach et al. 2005; Rosengrave et al. 2008; Butts et al. 2012). Although OF has yet to be implicated in intraspecific gamete-level mate choice in salmonids (Evans et al. 2013), it has been shown to promote fertilization by conspecific sperm when in competition with those of sister species (Yeates et al. 2013). Intriguingly, however, there is evidence from an internally fertilizing poeciliid fish that OF within the female’s reproductive tract can selectively bias fertilization in favor of sperm from unrelated males over related males (Gasparini and Pilastro 2011). Recent work on an externally fertilizing wrasse has also shown that OF can bias competitive fertilization success toward dominant “nest” males (i.e., directional cryptic female choice; Alonzo et al. 2016). Our findings for mussels complement these prior studies by showing that egg chemoattractants similarly play an important role in mediating intraspecific sperm competition, thus exposing a previously unforeseen mechanism of sexual selection that may occur more broadly in other taxa. We suggest that further investigation into the effects of female reproductive fluids, including egg chemoattractants, across a broader range of taxa will provide fruitful mechanistic insights into gamete-level mate choice.

We also found that the competitive fertilization biases induced by egg chemoattractants reflect complex genetic relationships between the focal males and focal (i.e., chemoattractant producing) females. These results may shed some light on patterns of genetic compatibility that underlie competitive fertilization biases, given previous findings that differential chemotaxis is correlated with offspring fitness of male–female pairs (Oliver and Evans 2014). Competitive fertilization success was higher for focal males that had a lower overall genetic relatedness to focal females (based on neutral nuclear markers), which complements recent evidence in other taxa that preferences for genetically dissimilar males may drive compatibility-based cryptic female choice (Gasparini and Pilastro 2011; Firman and Simmons 2015). Although we did not directly examine the extent of inbreeding in our population, homozygote excesses consistent with inbreeding are not uncommon in populations of broadcast spawners (Huang et al. 2000; Addison and Hart 2005; Kenchington et al. 2006), possibly due to the unpredictable patterns of spawning and recruitment in these systems (Hedgecock and Pudovkin 2011). Therefore, gamete-level mechanisms of maximizing offspring heterozygosity may be important for individual reproductive fitness.

In contrast to the patterns of overall genetic relatedness, we also found a competitive fertilization bias toward males that had the same phylogenetic mitochondrial lineage as the female. Preferences based on phylogenetic lineage are not unexpected in Australian *M. galloprovincialis* populations, as Northern and Southern Hemisphere lineages had diverged in allopatry from the Pleistocene before the more recent introduction of Northern individuals (Hilbish et al. 2000; Gérard et al. 2008). Nevertheless, it appears that such preferences have not maintained reproductive isolation between lineages, with the admixture of nuclear genotypes in our population supporting previous findings for Australian populations (Westfall and Gardner 2013). Possibly, this could be due to lineage-based patterns being offset by the preferences for less related nuclear genotypes. However, the precise fitness benefits of the mitochondrial lineage-based biases deserve further investigation. For example, one possibility is that fertilization biases reflect cyto-nuclear compatibilities brought about by the presence of divergent mitochondrial lineages; it would therefore be interesting to examine how preferences relate to nuclear genes involved in mitochondrial function. Moreover, we sequenced the female-type mtDNA common to somatic tissues of both males and females, but the occurrence and transmission of male-type mitochondria in sperm may further complicate patterns. Therefore, the precise genetic interactions between males and females that underlie chemoattractant-driven fertilization biases in these systems remain to be fully resolved.

To provide further mechanistic insights into gamete-level mate choice in this system we need to identify the chemical profiles of egg chemoattractants and determine how variation in these profiles correspond to patterns of differential sperm attraction. Chemoattractant molecules have not yet been identified in *M. galloprovincialis*, but several types of egg-derived chemicals have been described in other broadcast spawners (reviewed in Evans and Sherman 2013). For example, in echinoderms, peptides released from eggs bind to guanylyl cyclase receptors on the sperm surface, triggering a signaling pathway that results in influxes of extracellular calcium ions and a corresponding flagellar beat pattern (Kaupp et al. 2006; Alvarez et al. 2014). However, to our knowledge there has been no examination of intraspecific variation in such signaling pathways in any species. Recent evidence suggests that sperm-activating peptides are evolutionarily conserved and vary little within genera (Jagadeeshan et al. 2015). Therefore, it may be unlikely that a single molecule type (such as a particular peptide) is responsible for intraspecific variation in sperm chemoattraction. Instead, it is possible that eggs release a variety of molecules that affect such signaling pathways. Our finding that the interacting effects of parental genotypes drive chemoattractant preferences suggests that these chemical signals are likely to be complex. Clearly there is a need to characterize

intraspecific variation in egg chemoattractant chemical profiles to address these questions.

In conclusion, we provide the first direct evidence that egg chemoattractants moderate sperm competition and complement these findings with genetic data that may explain the previously documented offspring fitness benefits associated with differential sperm chemotaxis (Oliver and Evans 2014). Given our focus on a species exhibiting the ancestral mating strategy of broadcast spawning, and the fact that egg chemoattractants are found throughout a diverse range of taxa (Miller 1985; Eisenbach 1999; Teves et al. 2009), we anticipate that such mechanisms of gamete-level mate choice may be prevalent in other species. However, until now the putative role of sperm chemotaxis in mediating intraspecific sperm competition has been largely untested. This is likely due in part to the empirical difficulty of linking the effect of putative mechanisms of gamete-level mate choice directly to variation in competitive fertilization success. We demonstrate that powerful and tightly controlled experimental designs can provide detailed insights into the intricacies of gamete-level sexual selection.

#### AUTHOR CONTRIBUTIONS

R.A.L., W.J.K., and J.P.E. designed the study. R.A.L. conducted the experiments and data collection. All authors discussed analysis of the data, and R.A.L. performed the formal statistical analyses. R.A.L. wrote the first draft of the manuscript, and all authors contributed to the final version.

#### ACKNOWLEDGMENTS

We thank Jukka Kekäläinen for assistance with mussel collections, Stephen Robinson and Catherine Seed for helping design and create the chemotaxis chambers, Yvette Hitchen for assistance with microsatellite and mtDNA analyses, and Alan Lymbery and two anonymous reviewers for helpful comments on the manuscript. Funding was provided by the UWA School of Animal Biology (R.A.L.) and the Australian Research Council (J.P.E.; Grant Number DP150103266). R.A.L. was supported by the Hackett Postgraduate Research Scholarship and the Bruce and Betty Green Postgraduate Research Top-Up Scholarship.

#### DATA ACCESSIBILITY

Fertilization success, genetic relatedness, and mitochondrial lineage data associated with this study have been deposited with Dryad (DOI: <https://doi.org/10.5061/dryad.4kh60>). Novel mitochondrial DNA sequences (haplotypes H8, H10, H11, and H12) have been deposited in GenBank with the accession codes MF462182, MF462183, MF462184, and MF462185.

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Associate Editor: R. Snook

### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

**Table S1.** Primer sequences, size range (Bp, base pairs) expected from literature (observed size range in parentheses) and references for the 13 microsatellite loci for *M. galloprovincialis* used in this study.

**Table S2.** Tests for null alleles at 13 microsatellite loci for *Mytilus galloprovincialis*, performed with Bonferroni correction for multiple tests using MICROCHECKER.

**Table S3.** Mean and standard error of maximum likelihood genetic relatedness of focal male – focal female pairs, estimated from all 11 microsatellite markers used in the final analysis and combinations with each marker removed in turn.

**Table S4.** F-type CO1 haplotypes of *Mytilus galloprovincialis* recorded in our study.

**Table S5.** Link-scale approximation of variance components associated with random effects in the full generalized linear mixed model of competitive fertilization success.

**Table S6.** Comparison of models with different combinations of the fixed effects nuclear genetic relatedness, mitochondrial lineage and their interaction on competitive fertilization success.

**Figure S1.** Bayesian modelling of subpopulation structure in microsatellite data, comparing the probability of models with different numbers of clusters ( $K = 1–5$ ).

**Figure S2.** Bayesian phylogenetic tree for *Mytilus* spp. female-type CO1 mitochondrial DNA haplotypes, rooted in *M. trossulus* haplotypes.

Supplementary Methods: Sperm dye contamination trials; Testing for subpopulation structure of nuclear genotypes; Comparing performance of genetic relatedness measures.