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Female reproductive fluid and post-mating sexual selection in two fish species with external fertilization

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Thesis abstract

Post-mating sexual selection is the consequence of polyandry (i.e., female promiscuity), a taxonomically widespread phenomenon that causes the ejaculates of different males to overlap in time and space and compete to fertilize the same set of eggs. Sperm competition has been traditionally considered the post-mating equivalent of male competition, and hence regarded as an intra-sexual process. However, the arena in which sperm competition occurs is largely shaped by the female reproductive environment, enabling females to interfere with, and potentially control, the outcome of sperm competition, a process known as cryptic female choice. Female reproductive fluid (the fluid that surrounds the eggs before and during fertilization, hereafter FRF) has recently been identified as a mediator of cryptic female choice in both internal and external fertilizers, for its ability to differentially influence ejaculates of different males and bias fertilization towards specific partners. In particular, the frequent occurrence of these processes in external fertilizers makes them the ideal model to explore the female influence over sperm competition. External fertilization can indeed help to disentangle male- from female-driven effects and study their relative importance in determining the outcome of competitive fertilizations.

In this thesis, to gain a broad perspective of the role of FRF in post-mating sexual selection, I employed two externally fertilizing fish species with different reproductive systems: the zebrafish *Danio rerio* and the grass goby *Zosterisessor ophiocephalu*s. In the zebrafish both pre- and post-mating competition between males (and ejaculates) are rather common, often resulting in multiple paternity in the clutches and limited female pre-mating choice. The grass goby is characterised by territorial-sneaker male reproductive tactics and a strong female pre-mating preference towards territorials, hampered by sneakers’ attempts to parasitize the spawning events, leading to intense sperm competition and multiple paternity in the clutches. In both species, cryptic female choice is therefore expected to play a key role in determining the reproductive outcome, enabling females to gain some control over the fertilization process in case of the zebrafish, and to reinforce their pre-mating preference for territorial males in case of the grass goby.

The research conducted with the zebrafish uncovered novel cryptic choice mechanisms of FRF, acting on the gametes of both sexes. The results of paper I revealed the potential of FRF to increase multiple paternity in the offspring by prolonging the egg fertilization window, thus expanding the opportunities for sperm competition and for exerting cryptic female choice. The mechanism by which females of the zebrafish influence sperm competition through their FRF was investigated in

paper II, where the change in sperm velocity caused by FRF was found to directly predict the change in paternity share between rival males from water to FRF, providing the first evidence of the direct association in direction and magnitude of FRF phenotypic and fitness effects.

With the grass goby, I explored the potential for a FRF-mediated directional cryptic choice towards the territorial phenotype, by investigating the previously unexplored effects of FRF concentration and interaction with male seminal fluid. The findings of paper III revealed a differential concentration-dependent effect of FRF over sneaker and territorial sperm performance, favouring sperm depending on their proximity to the eggs during spawning. Such a mechanism was found to favour territorial males, but likewise advantage sperm of those sneakers able to get close to the eggs, allowing flexibility in the direction of female post-mating choice. Finally, in paper IV, our findings revealed an intrinsically differential effect of FRF on the fertilization success of the different tactics, mediated by its interaction with male seminal fluid. By interfering with the impairment of sperm competition caused by the seminal fluid in this species, FRF was indeed shown to rebalance the competition among ejaculates favouring territorial males, thus reinforcing female pre-mating choice.

Ultimately, the combined results of this thesis, provide a key indication of the powerful selective role of FRF in the post-mating scenario, revealing new ways by which females can tailor their influence over the ejaculate competition system.

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1. INTRODUCTION

Sexual selection: a never-ending debate

Few topics in evolutionary biology have been as rich in controversies and alternative explanations as Charles Darwin’s theory of sexual selection. Originally, sexual selection was proposed to explain the evolution of traits that could not have evolved by natural selection, as do not increase survival or fecundity (Andersson; Shuker & Kvarnemo, 2021). Those traits are what today we call secondary sexual traits, which differ between males and females but do not play a direct role in reproduction. As Darwin first pointed out, these traits often present an extreme sexual dimorphism and tend to be much more exaggerated in males, that use them during the mating season to fight with other male competitors or to court females (Andersson, 1994). According to Darwin’s original formulation (1871), sexual selection “arises from differences in reproductive success caused by competition over mates” and depends “...on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring” (Darwin, 1859). The focal point of this definition seems to be on the competition between the males, but the theory is made of two components: an intra- and an inter-sexual selection, most commonly expressed by male-male competition and female choice.

In male-male competition there is a direct interference between the male competitors for access to the females or to critical resources needed by the females, and this drives the evolution of the traits we call armaments, or weapons, (e.g., antlers, horns, shields, badges of status…). However, the extravagant male traits that captured Darwin’s attention in the first place (colour patterns and patches, courtship displays, vocal calls…) don’t usually play a role in the direct competition between males, but rather serve the purpose of attracting females and persuading them to mate (Andersson, 1994; Emlen, 2008). The evolution of such traits involves inter-sexual selection, the process called female choice (i.e., the mate choice performed by females on the potential male partners).

The essence of Darwin’s argument for female choice lies in the idea that, in presence of variation among males with respect to their secondary sexual traits, it seems unlikely that females could mate randomly and show no preference whatsoever. In the *Descent of man* (1871), he states that “…when we see many males pursuing the same female, we can hardly believe that the pairing is left to blind chance—that the female exerts no choice and is not influenced by the gorgeous colours or other ornaments with which the male alone is decorated’’. It follows that even a weak preference for some of these male traits would be enough to trigger differences in reproductive success and, therefore, for sexual selection to operate (Jones & Ratterman, 2009).

If on one hand the idea of competition among males has been widely accepted by Darwin's contemporaries, not least because of the self-evidence of male combat in species carrying extremely conspicuous weapons (Andersson, 1994; Emlen, 2008; Shuker & Kvarnemo, 2021), the concept of mate choice was at first dismissed as inconceivable. The main reason behind this refusal can likely be found in the strong cultural bias of the Victorian age: Darwin put the emphasis on female mate choice in a historical time when it was not common to consider women as equal to men, let alone accept that they could be in charge of such an important choice. Yet, this was not the only issue with the mate choice idea: the idea of "preference" as described by Darwin gave the impression of a human-like sense of aesthetics, suggesting that a certain level of "intelligence" and "mental powers" (Darwin, 1871) would be needed to appreciate beauty (Jones & Ratterman, 2009; Prum, 2012). It took nearly a century to overcome this anthropocentric view of mating preferences, yet at the same time the resurgence of interest in the topic during the 20th century brought forth controversy. Mate choice was starting to be recognized as a powerful evolutionary mechanism, mostly thanks to ground-breaking empirical and theoretical studies (Lande, 1981; Andersson, 1982; Kirkpatrick, 1982), but the mechanisms involved in the evolution of mate preferences emerged as a challenging topic.

Choosing a partner carries a cost, may it be higher exposure to predation risk, or less time available for foraging, or the energy spent in travel and assessment time, and must therefore come with benefits for the choosy sex. In those species where males provide resources that increase females' survival and reproductive success (such as nuptial gifts, food, territories, parental care…), the so-called direct benefits, the rewards of mate choice are relatively straightforward to comprehend (Andersson, 1994; Møller & Jennions, 2011). Direct benefits cannot, however, explain the evolution of mate preferences for most of the spectacular and extreme ornaments observed in the animal kingdom. In non-resource-based mating systems males appear to offer nothing other than sperm to females and genes to the future offspring, making it much more challenging to establish how mating preferences evolved. Therefore, thanks to the newly acquired knowledge of the genetic mechanisms underpinning Darwin's theory, various indirect benefits models, not necessarily mutually exclusive, have been proposed to fill this gap.

The first explanation to be proposed was the Fisher's runaway model, formulated in the 1930s by the population geneticist R.A. Fisher and later mathematically formalized (Fisher, 1930; Lande, 1981, Kirkpatrick, 1982). According to the Fisher’s runaway model, if females with a preference for a specific male trait tend to mate with males carrying that trait, then the genes for the female preference and the male trait are going to be in linkage disequilibrium in their offspring. The trait and the preference are therefore bound to coevolve, in a “runaway” process that favours the exaggeration of both. To date, a direct observation of this runaway process is still lacking. Another, more successful, model for the evolution of mating preferences is the "good genes" model, according to which some male traits or displays represent reliable indicators of the male overall genetic quality and are correlated with other traits that increase lifetime fitness (Andersson, 1994; Kirkpatrick, 1996). Since these traits can sometimes be extreme and costly to maintain, males able to survive carrying such ornaments, or performing spectacular displays, are supposed to be in good shape and of higher genetic quality. This model has proved to work for specific traits, such as for instance the red/orange coloration, based on carotenoid pigments, that are obtained by food and play a crucial role in the effectiveness of the immune system (e.g., the three-spined stickleback – Mehlis *et al.*, 2013), but its sometimes too generous application is still debated today. “Good genes” being the default explanation for the evolution of costly elaborate ornaments, in fact, goes against ample evidence indicating that the most attractive mates often are not the ones producing the most viable offspring, and other indirect benefits, including genetic compatibility, heterozygosity, and offspring attractiveness are likely involved (Achorn & Rosenthal, 2020).

Today we know that another possible mechanism for the evolution of mate preferences is natural selection acting on pleiotropic effects of genes that affect mate choice, resulting in perceptual biases. In these cases, the female preferences are by-products of preferences unrelated to reproduction, that evolved before the male signals were even present (e.g preferences for food of a specific colour, or for sounds that mimic those produced by usual preys; Ryan & Cummings, 2013). Concurrently with the blossoming of these new models another issue arose: the paradox of the maintenance of genetic variation in male secondary sexual traits despite the strong directional selection acting on them through female choice (also known as the “lek paradox”). Several resolutions have been proposed over the years, notably the host-parasite coevolution hypothesis (Hamilton & Zuk, 1982) and the genic capture hypothesis (Rowe & Houle, 1996), with empirical work mostly focusing on the relationship between male traits and genetic benefits for the offspring (Qvarnstrom, 2001), but it’s going to be only thanks to the discussion revolving around post-mating sexual selection and compatibility that the paradox will be further cleared up.

As summarized in this introductory chapter, sexual selection represents a fundamental piece of our biological thinking, subject to continuous revolution and able to cause a spirited debate among scholars for more than a century. To date, the debate around the topic is all but settled to the point that in 2021 a new definition of sexual selection, combining different elements from the previous, was proposed: *"Sexual selection is any selection that arises from fitness differences associated with non-random success in the competition for access to gametes for fertilization"* (Shuker & Kvarnemo, 2021).

This definition results from the century-long debate about sexual selection and how to distinguish it from natural selection, building on a pre-existing emphasis on differential access to fertilization, already present in:

- Kokko *et al.,* 2006: "sexual selection is the selection generated by differential access to opposite-sex gametes (or mates)."
- Kuijper *et al.,* 2012: "sexual selection is the process by which individuals compete for access to mates and fertilization opportunities."
- Safran *et al.,* 2013: "sexual selection is the result of the differential reproductive success that arises from competition for mates and access to fertilizations."

These examples evidence the inherent complexity of providing a general but precise definition of sexual selection, without tiding it to a specific mechanism (like competition, that is still present in the definitions of Kuijper *et al.,* 2012 and Safran *et al.,* 2013), while also being able to encompass all the scenarios where a selective force arising from fitness differences operates.

The first thing that stands out is that these new definitions are agnostic as to the sexual identity of the competitors, a consequence of the heated discussion on the Darwinian sex roles that characterized the recent debate. Even though Darwin was already able to appreciate that sex roles could occasionally be reversed, and that sexual selection could therefore operate on females rather than on males, most of the experimental research has undoubtedly been done on males. Furthermore, Darwin's initial characterisation of males as eager to mate and competing for access to the females, and females as choosy and passive (Darwinian sex roles), received a substantial corroboration in 1948, when Bateman experimentally demonstrated the different strength of sexual selection in the two sexes (reported by Trivers in his theoretical formulation in 1972). Specifically, he showed that in the fruit fly *Drosophila melanogaster* the reproductive success (i.e., number of offspring produced) of males increased with the number of females he copulated with, while female fecundity did not change after the first copulation partner. He therefore concluded that promiscuity seemed to be beneficial for males in terms of offspring quantity, but not for females, that could only hope to increase their offspring's quality by choosing the right partner.

The ultimate reason behind this difference lies in the different investment in gametes between males and females, due to disruptive selection on gamete size and number, leading to males producing a lot of small and mobile sperm, and females producing few large, immobile eggs (Trivers, 1972; Parker *et al.,* 1972; Gage & Morrow, 2003).

Bateman's conclusions, together with Darwin's behavioural observations of males and females of various species, became known as the Darwin-Bateman paradigm. Under this paradigm, females played a crucial role as selectors of male quality but were typically considered to be strictly monogamous and not under substantial selection pressure themselves (Hare & Simmons, 2019).

The overcoming of this idea led to the second novel component of the modern definitions of sexual selection: competition is for access to *gametes*, not to partners, of the opposite sex. This was only possible through a thorough review of the female perspective of sexual selection and the realization that females of most species are far from monogamous and may even actively seek multiple partners (Pizzari & Birkhead, 2002). The biological implication of polyandry (i.e., female promiscuity) is the possibility for sexual selection to persist after the mating event, up to the point of fertilization and, occasionally, even beyond (Pizzari & Birkhead, 2002; Tregenza & Wedell, 2000).

The more the merrier: polyandry and post-mating sexual selection

The polyandry revolution stimulated a major turning point in the field of sexual selection, with Parker's review (1970) of sperm competition in insects stating, for the first time, that females mating with multiple partners in the same reproductive episode would lead to the overlap, and therefore the competition, between ejaculates of rival males. Once again, it was only with a certain delay that the female side of post-mating sexual selection started to draw attention. The phenomenon called "cryptic female choice" was described for the first time in 1983, when Thornhill provided the experimental evidence that females can bias the fertilization success of the male competitors they copulate with (Thornhill, 1983). However, it was not until 1996, when Eberhard's monograph on cryptic female choice came out, that the scientific community became aware of the female potential to exert post-mating control over the outcome of sperm competition.

Nowadays, polyandry is widely held to be a taxonomically widespread phenomenon (Birkhead & Moller, 1998; Simmons, 2001; Griffith *et al.,* 2002), but at first it was dismissed as irrelevant and thought to be a mere by-product of the positive selection for promiscuity genes acting on males (Birkhead & Pizzari, 2002). In light of the Darwin-Bateman paradigm, indeed, the benefits of mating with multiple partners are extremely intuitive for males, but not for females, which produce a limited number of costly gametes and cannot so much increase their reproductive success with additional copulations. Subsequent research in the field shed light over the many benefits females can gain by mating promiscuously, describing both direct and indirect benefits (see table 1 for a comprehensive list). Direct benefits include increased resources or protection for themselves and their offspring, or fertility gains secured through an augmented sperm supply, a key insurance against the first male being sterile or sperm depleted (Petrie, 1992; Levitan, 1998; Clutton-Brock & Vincent, 1991; Birkhead & Pizzari, 2002; Parker & Birkhead, 2013).

Indirect benefits, on the other hand, are deeply connected to the cryptic choice process and provide females with advantages related to the quality of their future offspring. By mating with additional partners females can indeed increase their chances of finding better or more genetically compatible partners and bias fertilization towards them, thus exerting cryptic female choice (Jennions & Petrie, 2000; Tregenza & Wedell, 2000; Zeh & Zeh, 2001; Parker & Birkhead, 2013).

Table 1. Potential benefits of polyandry (references: Petrie 1992; Levitan 1998; Clutton-Brock & Vincent 1991; Jennions & Petrie, 2000; Tregenza & Wedell, 2000; Zeh & Zeh, 2001; Birkhead & Pizzari, 2002; Parker & Birkhead, 2013).

DIRECT BENEFITS	INDIRECT BENEFITS
Resources acquisition: nutrients through courtship or nuptial gifts, protection and/or paternal care	**Offspring genetic diversity:** higher genetic diversity in the offspring increases their chances of survival
Fertility benefits: multiple copulations ensure an adequate sperm supply (avoid sperm limitation) and increase the proportion of fertilized eggs	**Offspring viability:** when multiple ejaculates overlap females can select the most viable sperm and produce viable offspring
Partner change: additional copulations potentially permit to obtain a better partner and reduce the cost of choosing the “wrong” male	**Offspring attractiveness:** when multiple ejaculates overlap females can select the most “attractive” sperm and produce sons with attractive sperm
Limit cost of harassment: resisting extra mating attempts can sometimes be more costly than accepting them	**Genetic compatibility:** multiple copulations increase the chances of finding the most genetically compatible sperm/partner

Darwin's oversight of post mating sexual selection was only in part due to a distorted view of the female sex role, another important part was played by his dismission of what he called the "lower" classes, considered to be unaffected by sexual selection (Darwin, 1871). In doing so he failed to recognize the strong selective force that competition among ejaculates exert on the sessile and weakly mobile organisms with external fertilization (e.g., marine invertebrates that exhibit the reproductive strategy of broadcast spawning; Levitan, 2010; Parker *et al.,* 2018).

Interestingly, such organisms and their spawning strategies played a major role in the succession of evolutionary events (the "sexual cascade") that starts with anisogamy and culminates in the rise of pre-mating sexual selection (Parker, 2014). The post-mating stage of sexual selection indeed evolutionary preceded the pre-mating one, and constitutes the ancestral step of the cascade, followed by the emergence of specific features, such as increased mobility, behavioural complexity, and internal fertilization, that ultimately resulted in pre-mating sexual selection, and the consequent higher differentiation of female and male sexual strategies (Parker, 2014; Parker & Pizzari, 2015). Broadcast spawning and, more in general, external fertilization therefore have the potential to offer unique insights into the evolutionary transitions of sexual selection.

Anisogamy, being the cornerstone of the sexual cascade, also lays the foundation for sexual conflict, defined as conflicting evolutionary interests of the sexes arising from sexual selection when a mutation (termed sexually antagonistic mutation) causes changes increasing fitness in one sex, but decreasing it in the other (Sharer *et al.,* 2012). It was only in the face of the new awareness of post-mating sexual selection, stimulated by the polyandry revolution of the 1970s, that the scientific community started to appreciate that male and female interests could often conflict and that this could generate sexually antagonistic coevolution (Parker, 2014). Such a conflict between the sexes ultimately concerns the control over the fertilization outcome, with males' adaptations favouring their own sperm in the ejaculate competition or favouring the ability to prevent females from copulating with additional partners, driving females' responses to regain post-mating control and sharpening mechanisms of sperm selection, in a continuous arms race process.

Finally, it is worth mentioning that sperm competition and cryptic female choice are not the sole manifestations of post-mating sexual selection. Indeed, when sex roles are reversed (e.g., in the pipefish *Syngnathus typhle*; Berglund, 1991) and under conditions of sperm limitations in broadcast spawners (e.g., the polychaete *Galeolaria caespitosa;* Marshall & Evans, 2005) competition among eggs may occur, with selection acting on female traits (e.g., egg size) that increase their competitive success.

In addition, even though this thesis will continue to focus solely on the animal kingdom, it should be noted that many of the mechanisms of sexual selection discussed here, primarily those operating after gamete release, are applicable also to other taxa, like plants and fungi (Moore & Pannell, 2011; Beekman *et al.,* 2016).

With the rise of post-mating sexual selection research, studies on the topic have initially focused only on the gametes (and mostly on sperm), neglecting both the non-gametic components and the interaction between the sexes, under a separatist idea of distinct processes of intra- (sperm competition) and inter-sexual selection (cryptic female choice). It was only recently that it was demonstrated that the non-gametic components released with the gametes play an important part in this stage of sexual selection (reviewed in: Pitnick *et al.,* 2020; Ramm, 2020; Gasparini *et al.,* 2020). Unsurprisingly, the male non-gametic component (seminal fluid) was the first to raise interest in the context of post-mating sexual selection, as will be discussed in the next chapter, while female reproductive fluids remained completely overlooked, and with them the potential for fluids interaction.

Sperm (and ejaculates) competition

The theoretical formalization of sexual selection through sperm competition was originally conducted by Geoff Parker (1970), in an influential review highlighting the existence and evolutionary significance of such a process.

His original definition of sperm competition was "the competition within a single female between the sperm of two or more males for the fertilization of the ova", later broadened to "the competition between the sperm of two or more males for the fertilization of a given set of ova" to include also externally fertilising species (Wigby & Chapman, 2004). The corpus of theoretical and empirical research addressing the issue of selective outcomes of sperm competition generated the prediction of two opposite selective pressures acting on males: one favouring traits that prevent, or at least minimise, the chances of competition with rival ejaculates, and one favouring traits that enable males to outcompete the rival ejaculates.

The male strategies to avoid direct sperm competition are mostly directed at preventing females from mating with rivals. One of the most widespread male adaptations is behavioural mate guarding, which can occur before or after copulation (Parker, 1970; Birkhead, 1985; Alonzo & Warner, 2000). For instance, males of some species where first male sperm precedence occurs, or where female sexual receptivity is time-restricted (e.g., in crustaceans; Jormalainen, 1998) might try to conceal their mates from male competitors before copulation. Conversely, in species with last male sperm precedence post-copulatory mate guarding is prevalent (Wigby & Chapman, 2004). A common variation of this behaviour is the extension of copulation duration, as a way to exclude rival males until the end of the female receptive period, or the eggs deposition (Parker, 1970; Birkhead & Moller, 1998). Aimed at preventing females from mating with other males is also the application of mating plugs, formed by coagulation of the ejaculate or by diverse substances produced by accessory glands. These plugs mechanically block the entrance to the female reproductive tract after copulation has taken place, interfering with female polyandry while leaving the male free to pursue additional matings, and have been documented in a wide range of animal taxa, including mammals, birds, reptiles, amphibians, insects and arachnids (Simmons, 2001; Uhl *et al.,* 2010; Simmons & Fitzpatrick, 2012). In spiders, for instance, mating plugs can consist of either secretory, amorphous material or of parts of the males’ pedipalps (i.e., copulatory organs), that are left behind in the female genital tract (Uhl *et al.,* 2010). Additionally, males can also prolong female post-mating sexual receptivity and remating time through injuries to the female reproductive tract or specific molecules transferred with the ejaculate (Stockley, 1997; Birkhead & Hunter, 1990). Finally, when a female has already mated with rival males, an additional strategy to avoid, or at least minimize sperm competition, is the displacement of the ejaculates of previous males from female storage organs or reproductive tract (Simmons, 2001; Wigby & Chapman, 2004).

Nevertheless, often sperm competition cannot be avoided and hence traits that enable ejaculates to outcompete those of rivals should be favoured by sexual selection. Ejaculate investment was undoubtedly the first trait explored in this context, with early predictions positing that in situations of intense sperm competition it would benefit males to increase the expenditure in sperm production (Parker, 1970; Parker, 1984). Consistently with this prediction, empirical evidence from a wide range of taxa showed that species experiencing higher levels of sperm competition had relatively large testes (Birkhead & Moller, 1998; Hosken & Ward, 2001).

Regardless of their investment in sperm production, all males might experience sperm depletion between successive matings, and are therefore expected to allocate sperm strategically (Birkhead & Pizzari, 2002). Strategic sperm allocation theories predict that in presence of moderate sperm competition, males should allocate ejaculates that contain more sperm. When the competition intensifies, which is a common occurrence for some externally fertilising fish, it pays more to invest fewer sperm per single ejaculation instead, as the chances of fertilization are already reduced (Stockley *et al.,* 1997; Parker, 1984).

Sometimes, the sperm allocation in the ejaculate can be modified strategically only to some extent, like in the case of species with alternative reproductive tactics, where the sperm expenditure strategy is fixed by the male's tactic (Mazzoldi *et al.,* 2000; Rasotto & Mazzoldi, 2002; Neff *et al.,* 2003; Rudolfsen *et al*., 2006). These species constitute an excellent model for the study of sperm competition dynamics, as the alternative male phenotypes usually experience different levels of sperm competition: the dominant (territorial/nesting/guarding) controls the majority of matings or resources and is subject to variable levels of competition, according to the number of opportunistic (sneaker/satellite/parasitic) males present (Taborsky, 2001). These, in turn, always experience competition with at least one male, the dominant one, and have accordingly been shown to invest relatively more in sperm compared to the tactic experiencing lower risk (Oliveira *et al.,* 2008; Petersen & Mazzoldi, 2010).

However, it became evident that the sperm number alone could not explain the variance in paternity success observed among males of numerous species (Parker & Pizzari, 2010; Immler *et al.,* 2010; Simmons & Fitzpatrick, 2012). Hence, research effort moved towards the evolution of individual sperm traits such as sperm swimming velocity, motility, viability and/or longevity, size and morphology (collectively referred to as sperm quality; Snook, 2005). All these traits were indeed known to vary considerably among males and started to attract attention for their potential relation with the level of sperm competition, leading to varying results. As for sperm velocity and motility (i.e., the proportion of motile sperm cells over the total cells in the ejaculate), a positive relation was shown between these traits and fertilization success (reviewed in Simmons & Fitzpatrick, 2012), with comparative studies showing that species subject to intense sperm competition often have a higher proportion of motile, faster swimming, sperm (Gage *et al.,* 2004; Pizzari & Parker, 2009; Lupold, 2013). There are only very few exceptions to this pattern, with slower sperm being more competitive in some species because better at achieving successful fertilization (in the frog *Crinia georgiana,* Dziminski *et al.,* 2009) or at being stored by females (in *Drosophila melanogaster,* Lupold *et al.,*2012). Sperm viability (i.e., the proportion of live sperm cells able to fertilize an ovum; Zegers-Hochschild *et al.,* 2017) and/or longevity (i.e., the amount of time that sperm can remain alive and capable of fertilizing an ovum; Zegers-Hochschild *et al.,* 2017) are also included in the set of male adaptations to sperm competition (Snook, 2005). Evidence from insects (Hunter & Birkhead, 2002) and birds (Rowe & Pruett-Jones, 2011; Smith, 2012) have positively linked sperm viability to sperm competition, while sperm longevity was suggested to be an important predictor of paternity under natural spawning conditions of asynchronous gametes' release (Gage *et al.,*. 2004).

Finally, the relation between sperm competition and sperm size is inconsistent among taxa, with seemingly no widespread evidence of the long-held assumption that velocity might be directly related to sperm length (Humpries *et al.,* 2008; Laugen *et al.,* 2022). There appears to be instead a pattern related to the different mating systems: the relationship between sperm velocity and the sperm head-flagellum length ratio has been found to be positive for internal fertilizers and negative for external fertilizers (Fitzpatrick & Lupold, 2014; Simpson *et al.,* 2014). Additionally, individual males of many species show considerable variation in sperm morphology within their ejaculates (Birkhead & Pizzari, 2002). In *Drosophila pseudoobscura* for instance, males produce two or more sperm sizes, in lepidopterans and molluscs both nucleate and anucleate (non-fertilizing) sperm are produced, seemingly for a paternity defence purpose achieved through the filling of the sperm storage organs of females, that are therefore less likely to remate with other males (Snook & Karr, 1998; Cook & Wedell, 1999).

It clearly follows from this report that research on the male traits shaped by sperm competition focused almost exclusively on the sperm, be it production/number or quality. However, the original term employed by Parker (1970) to describe this phenomenon was "ejaculate" competition, initially misunderstood as only sperm, but later raising interest on the role of non-gametic ejaculate components as well. An increasing body of evidence points towards seminal fluids as important players of the sperm competition game, able to influence sperm competitiveness and females' behaviour (Pitnick *et al.,* 2020; Ramm, 2020). For instance, seminal fluids might influence the success of sperm in competitive situations by enhancing own sperm velocity (den Boer *et al.,* 2008; Holman, 2009; Simmons and Beveridge, 2011) or detrimentally affecting the sperm performance of rival males (Fry & Wilkinson, 2004, den Boer *et al.,* 2010; Locatello *et al.,* 2013). In presence of positive effects of seminal fluid on sperm performance, males of some species (mostly insects) can exploit rival ejaculates, and benefit from an equal improvement of sperm competitiveness (also called 'parasitism of rival ejaculates'; Hodgson & Hosken, 2006; Holman, 2009; Simmons & Beveridge, 2011). Additionally, seminal fluid components might affect female post-mating sexual receptivity and egg production, ultimately manipulating females' re-mating chances (Perry *et al.,* 2013; Pitnick *et al.,* 2020; Wigby *et al.*, 2020).

Despite the increasing knowledge of the mechanisms involved in sperm competition processes, the estimation of competitive fertilization success can often remain challenging, mostly because of an important, and neglected, confounding factor: the female reproductive environment. Sperm competition does not happen in a vacuum after all, but in an arena that can be largely shaped by the females to permit cryptic female choice, a process by which they can bias fertilization towards specific males, thereby interfering with and potentially controlling the outcome of sperm competition.

Cryptic female choice

As anticipated in chapter 1.2, starting from the end of the 1990s cryptic female choice, defined as any female-mediated morphological, behavioural, or physiological mechanisms that operate to bias fertilization toward the sperm of specific male(s), started to be acknowledged as a powerful engine of sexual selection (Thornhill, 1983; Eberhard, 1996; Firman *et al.,* 2017). From that time, evidence of female influence over the outcome of sperm competition has increased considerably, thanks to empirical research employing a wide range of taxa. Nevertheless, the mechanisms underlying cryptic female choice often remain unknown, mostly because of the experimental challenges associated with the observation of a process that necessarily requires the co-occurrence of sperm competition, and in internal fertilizers is hidden inside the female reproductive tract (Pitnick & Brown, 2000).

To prove cryptic female choice, it is not sufficient to pinpoint a female trait able to affect sperm utilization, but it’s crucial to demonstrate that the female-driven effect is differential, predictably favouring specific males over others because of their phenotype or genotype (Birkhead, 2000; Manier *et al.,* 2013; Firman *et al.,* 2017). Depending on the inherent characteristics of the males favoured through the process, cryptic female choice can result in directional or non-directional sexual selection. In order to have directional selection, fertilization needs to be biased in favour of specific male phenotypes, consistently preferred by the females, that in most cases correspond to those favoured in pre-mating choice, resulting in a reinforcement of the directional selection generated by female choice. Cryptic female choice is instead non-directional when female post-mating preferences differ among individuals and is directed to favour the sperm of the males with compatible genotypes, or of unrelated males, regardless of their phenotype, thereby working to maintain genetic variance and polymorphism (Birkhead & Pizzari, 2002; Firman *et al.,* 2017).

As previously discussed, cryptic female choice is likely to enhance the accumulation of those benefits achieved through polyandry by providing a selective environment for additive genetic benefits (fertilization success biased towards the competitively "best" sperm), or by fostering fertilizations by genetically compatible sperm (non-additive genetic benefits) (Simmons, 2005; Slatyer *et al.,* 2012).

The proximate mechanisms of cryptic female choice initially uncovered by empirical research are all unique to internal fertilizers and involve female differential responses at or shortly after mating (Eberhard, 1996). At mating, females might influence the paternity success of competitors by controlling the order and timing of different inseminations. In species with last male precedence, for instance, females can modulate the time between the first and successive matings thereby anticipating or postponing the time of displacement of the first male ejaculate (Xu & Wang, 2010).

Alternatively, females of some species can control the duration of the copulation event, directly affecting the amount of sperm inseminated by their mates (Pilastro *et al.,* 2007; Herberstein, 2011). In the guppy (*Poecilia reticulata*) for example, females allow attractive (brightly coloured) males to inseminate a higher amount of sperm through a longer copulation event (Pilastro *et al.,* 2004)

A mechanism of female post-mating control shared by highly taxonomically diverse internal fertilizers is differential sperm ejection/dump. In the feral fowl (*Gallus gallus domesticus*), for instance, females differentially expel ejaculates after multiple matings according to male social status, ejecting sperm from subdominant males when it is too costly to avoid their insemination attempts (Pizzari & Birkhead, 2000). Similarly, in the fruit fly (*Drosophila melanogaster*) females can exploit to their advantage the sperm displacement caused by additional matings after the first. By controlling the timing of ejection from the storage organs of displaced and excess sperm they can indeed affect the relative success of the male competitors and control to some extent the outcome of sperm competition (Lupold *et al.,* 2013). Cryptic female choice can also be exerted via different mechanisms of sperm uptake, such as contractions of the female reproductive tract, or orgasms-like responses, if these happens differentially and in response to specific male characteristics (see for example Troisi & Carosi, 1998; Friesen *et al.,* 2016). Finally, sperm attack by innate or acquired immune responses, phagocytosis, digestion and incapacitation (e.g. via spermicidal action in *Drosophila pseudoobscura*: Holman & Snook, 2008), inside the female reproductive tract are all pre-storage mechanisms able to create opportunities for cryptic female choice (Firman *et al*., 2017). If, instead, ejaculates manage to reach female sperm storage organs they have to face interaction with rivals and potential displacement, stratification, or mixing, and this seems to be related to the complexity of the female reproductive tract, with a higher number of storage organs associated with a better female control of paternity (Eberhard, 1996, Ward, 2000, Firman *et al*., 2017).

The processes of directional cryptic female choice and male responses can often lead to inescapable sexual conflict and to intersexual coevolution, and male evolutionary responses can meet or counteract female post-mating preferences. An example of the former can be found in the fruit fly *Drospohila melanogaster*, where directional selection promoted the evolution of giant sperm, in a coevolutionary dynamic where female seminal receptacles length and sperm length are genetically correlated, with longer receptacles selecting for longer sperm in a Fisherian-like process (Miller & Pitnick, 2002; Lupold *et al.,* 2012; Lupold *et al.,* 2016). Conversely, these dynamics can sometimes appear to be sexually antagonistic, like in waterfowl species, where male sexual coercion drove the evolution of complex female reproductive tracts, enabling females to retain control over fertilization, resulting in turn in an increased complexity of male genitalia as well (Brennan *et al.,* 2007).

What emerges from all these examples is the key role of the selective environment produced by the female reproductive tract in post-mating female control. The term “cryptic female choice” itself is related to the idea of a process hidden inside the female reproductive tract (Birkhead & Pizzari, 2002), so it’s no wonder that research in this field initially focused entirely on internally fertilizing species. For a long time, indeed, it was assumed that females of species with external fertilization could not exert any influence over the fertilization process, given that it does not take place inside the female body. Traditionally, in the face of these powerless females, post-mating sexual selection in external fertilizers was considered to be an exclusively male responsibility, only a matter of ejaculates in competition. However, it is precisely in species where females have limited control over males’ competition for fertilization, that mechanisms of sperm selection are particularly expected to be favoured by sexual selection, constrained to act almost exclusively at the level of gametic interactions (Evans & Sherman, 2013).

The pivotal role of external fertilization in post-mating dynamics of sexual selection becomes evident if we shift our perspective to the non-directional processes of selection that occur during gametes interactions. When the outcome of a competitive fertilization event seems to depend on specific male-female combinations, the explanation usually lies in different mechanisms of selection for genetic compatibility (Firman *et al.,* 2017). In different sea urchins’ species, for instance, external fertilization is mediated by the highly polymorphic (within species) sperm protein *bindin*, which variation leads to assortative fertilization: females' eggs indeed select sperm carrying a *bindin* genotype similar to their own (Stapper *et al.,* 2015). Similarly, egg glycoproteins in the house mice (*Mus musculus domesticus)* seem to mediate the selection of specific sperm genotypes, as a means of inbreeding avoidance, possibly through the recognition of specific major histocompatibility complex (MHC) haplotypes (Rulicke *et al.,* 1998; Ghaderi *et al.,* 2011).

Non-recombining regions of the genome like the MHC (a gene complex responsible for disease resistance and immune function) are prime candidates for the identification of partners' genotype from their sperm. Cryptic female choice mechanisms acting on MHC-based identification are expected to favour sperm of genetically dissimilar males or males with "optimal" MHC similarity, as a strategy to avoid inbreeding, or to maximize offspring heterozygosity and fitness (Milinski, 2006). MHC-dependent gamete fusion has been demonstrated also in both externally and internally fish (salmon - Yeates *et al.,* 2009; and guppies - Gasparini *et al.,* 2015).

Finally, very recent research has started to look also at the non-gametic components of the female reproductive environment, particularly at female reproductive fluids.

Female reproductive fluid

Unlike its previously discussed male counterpart (seminal fluid), female reproductive fluid has been largely neglected in the context of post mating sexual selection and has only recently been suggested to be a potential mediator of cryptic female choice.

The term female reproductive fluid (hereafter, FRF) refers to any medium, arising from females, through which sperm must pass on their way to fertilize eggs, inside the female reproductive tract in the case of internal fertilizers, or in the outside medium, where the FRF is released along with the eggs, for external fertilizers (Gasparini *et al.,* 2020). Such a broad definition accounts for the variety of components this fluid can be made of, and hence for the many different names that have been assigned to it across the animal kingdom. Components of FRF may include fluids of ovarian, follicular, oviductal and/or celomic origin, egg chemoattractants, cells from the peripheral surface of the eggs (e.g. egg jelly or cumulus cells; Gasparini *et al.,* 2020).

The primary role of FRF is to provide the appropriate environment for oocyte maturation, fertilization, and early embryo development (Leese *et al.,* 2001; Aguilar & Reyley, 2005). In external fertilizers FRF has been shown to prolong egg lifespan, protecting the eggs from the adverse environmental conditions in which they are released (Dietrich *et al.,* 2012). FRF is also involved in the protection of eggs from oxidative stress (Agarwal *et al.*, 2005; Da Broi *et al.,* 2018) and pathogens (Johnson *et al.,* 2014). Enzymes of antioxidant defence have been found in the FRF of various species, from insects (Baer *et al.,* 2009) to mammals (Harvey *et al.,* 1995; Fu *et al.,* 2016), and proteomic studies have revealed the presence of proteins related to the immune system in both internally and externally fertilizing species (Seppola *et al.,* 2009; Zamah *et al.,* 2015; Dosselli *et al.,* 2019). Interestingly, whether the abovementioned effects of FRF on the eggs could have implications for post-mating sexual selection has never been tested.

Amongst the naturally selected functions of FRF there is also the mediation of sperm chemotaxis, as evidenced from studies on marine invertebrates (Evans & Sherman, 2013), fish (Kholodnyy *et al.,* 2019; Zadmajid *et al.,* 2019), and mammals (Fabro *et al.,* 2002; Eisenbach & Giojalas, 2006), notably humans (Fitzpatrick *et al.,* 2020). Furthermore, increasing body of evidence highlights that FRF can significantly alter (generally improve) sperm behaviour, from sperm capacitation to sperm viability and longevity, swimming velocity and trajectory, all sperm traits typically involved in sperm competition and associated with fertilization success (Snook, 2005; Fitzpatrick & Lupold, 2014). FRF, indeed, was reported to trigger sperm capacitation and the acrosome reaction in mammals (Bravo & Valdivia, 2018), to increase the proportion of motile sperm after activation in externally fertilizing fishes (reviewed in: Zadmajid *et al.,* 2019) and the proportion of viable sperm in internally fertilizing insects (den Boer *et al.,* 2009; Liberti *et al.,* 2016). An extension of sperm longevity in presence of FRF has been described in a variety of taxa, both internally and externally fertilizing (fish - Gasparini & Evans 2013; Poli *et al.,* 2019; birds – Cramer *et al.,* 2016 and mammals – Zhu *et al.,* 1994; Sidhu *et al.,* 1999). Finally, positive effects of FRF on sperm velocity and trajectory have been observed in marine invertebrates (Evans & Sherman, 2013), amphibians (Simmons *et al.,* 2009), birds (Moller *et al.,* 2008), mammals (Oliveira *et al.,* 1999) and many fish species (see for a review Zadmajid *et al.,* 2019).

It was only very recently, however, that these effects of FRF on sperm traits started to be considered in the context of sexual selection. Accumulating evidence is indeed pointing at FRF as a mediator cryptic female choice, thus able to differentially affect sperm of different males and ultimately bias the outcome of sperm competition (Rosengrave *et al.,* 2016; Firman *et al.,* 2017, Gasparini *et al.,* 2020).

For example, FRF mediates sperm selection to avoid inbreeding by favouring unrelated males during fertilization in the guppy (*Poecilia reticulata*) (Gasparini & Pilastro, 2011), or towards the most compatible partner in the external fertilizer mussel (*Mytilus galloprovincialis*), where FRF has been shown to attract the sperm of the more genetically compatible male (Oliver & Evans, 2014). Also, FRF has been shown to mediate sperm selection towards the preferred male phenotype, and this seems to occur in species where males show alternative mating tactics, even though there is conflicting evidence on how this could work. In both the chinook salmon (*Oncorhyinchus tshawytscha*) (Lehnert *et al.,* 2017) and the masu salmon (*Oncorhynchus masou*) (Makiguchi *et al.,* 2016), for instance, FRF increases the sperm velocity of dominant males only, possibly acting on pre-existing differences in sperm velocity between the tactics (dominant males exhibit lower sperm swimming velocity). In the ocellated wrasse (*Symphodus ocellatus*), on the other hand, territorial males already have faster swimming sperm, and in this system FRF decreases the relative importance of sperm number over sperm velocity, thereby penalizing the numerical advantage of sneaker males (Alonzo *et al.,* 2016). Indirect evidence of FRF influencing sperm competition also come from studies reporting differential effects of FRF on the sperm performance of male competitors, like in the arctic charr (*Salvelinus alpinus*) (Urbach *et al.,* 2005), Chinook salmon (*Oncorhyinchus tshawytscha*) (Rosengrave *et al.,* 2008; Evans *et al.,* 2013), zebrafish (*Danio rerio*) (Poli *et al.,* 2019) and even humans (Fitzpatrick *et al.,* 2020).

Very little is known of the potential mechanisms behind FRF's effects on post-mating sexual selection. Studies on the compositional make-up of FRF have identified several chemical and biochemical components that could be responsible for sperm selection. Sperm performance was indeed shown to be influenced by FRF ionic composition (Na+, K+, Mg2+ and Ca2+) and macromolecules such as proteins, carbohydrates, lipids and female exosomes (Olson *et al.,* 2001; Kholodnyy *et al.,* 2019; Johnson *et al.,* 2020; Pitnick *et al.,* 2020; McCullough et al. 2022). FRF-specific proteins, like many glycoproteins, have been identified in insects, fish, and mammals (Lahnsteiner *et al.,* 1995; Aguilar & Reyley, 2005; Baer *et al.,* 2009), suggesting a specific function in the interaction with ejaculates (Gasparini *et al.,* 2020). Mechanisms underlying paternity biases through FRF may likely involve alterations to the physiology or structure of sperm, as shown in the mussel *Mytilus galloprovincialis*, where FRF can affect the acrosome reaction of specific males through modifications of the sperm surface glycans (Kekäläinen *et al.,* 2015; Kekäläinen & Evans, 2017).

Interestingly, studies on bees (den Boer & Boomsma, 2010) and ants (Dosselli *et al.,* 2019) highlighted the potential for interaction effects of FRF with male seminal fluid, showing that FRF in these species inhibits some serine proteases in the seminal fluid, thus increasing the viability of stored sperm. However, the implications of this interaction for post-mating sexual selection are still unknown, despite being very promising, particularly in the context of directional selection towards specific phenotypes, like in species with alternative mating tactics. Nor is there a clear indication of how the specific concentration of FRF encountered by different ejaculates might influence the dynamics of differential effects on sperm performance and fertilization bias previously described.

In conclusion, the potential of FRF to play a crucial role in sexual selection and particularly in female post-mating control have only recently emerged and brings with it a variety of outstanding issues and questions, making this an incredibly appealing field of research.

Aims of the thesis

The research carried out under my PhD project focused on the role of female reproductive fluid in the context of post-mating sexual selection, with the goal of expanding the female perspective of these processes to elucidate the role of female control over gametic interactions that have been typically described from a male evolutionary standpoint.

As evidenced throughout the theoretical background provided in the previous chapters, many open questions remain in the field of FRF-mediated cryptic female choice. During my PhD I have tried to tackle some of them using two different species of fish with external fertilization in order to gain a broad perspective of the role of FRF in post-mating sexual selection. The use of external fertilizers indeed allows to disentangle the effects of cryptic female choice from the ones driven by sperm competition and study their relative importance in determining the outcome of competitive fertilizations.

This thesis is structured as four paper chapters, each focusing on a different aspect of the FRF influence on post-mating sexual selection.

Paper 1 has the goal of unravelling the influence of FRF on the eggs, rather than on the sperm, and whether this might have implications for post-mating sexual selection, a topic that has been completely neglected so far.

Paper 2 focuses on the role of FRF in determining the outcome of competitive fertilization and on the relative strength of the different processes of sperm competition and cryptic female choice, by investigating the relationship between FRF effects on male post-mating traits (i.e., sperm performance traits) and the paternity distribution of rival males.

In **paper 3** we determine whether FRF can mediate a directional cryptic female choice towards the ejaculates of specific phenotypes, whether directly or through indirect mechanisms, investigating the hypothesis of a differential effect dependent on the FRF concentration encountered by the different ejaculates.

Paper 4 investigates the potential for FRF interaction with male seminal fluid in the context of directional choice, testing the hypothesis that FRF could play a role in rebalancing the competition among ejaculates to reinforce female pre-mating preferences at the post-mating level.

Finally, in the last chapter I provide a general discussion of the findings and conclusions from these papers and reason about the remaining gaps to fill, as well as the possible directions of future research.

The study systems: two externally fertilizing fish species

The two fish species employed in this project are the zebrafish *Danio rerio* and the grass goby *Zosterisessor ophiocephalus*. These two species have in common external fertilization and the frequent occurrence of sperm competition at spawning, but otherwise have rather different mating systems and are subject to different sexual selective pressures, hence allowing to deepen different aspects of the FRF-mediated post-mating sexual selection. The external fertilization facilitates to experimentally separate the gametic from the non-gametic components (male and female reproductive fluids) and study their different contributions to the selective processes in question, and most importantly to disentangle the effects of cryptic female choice from those driven by sperm competition. Furthermore, in externally fertilizing fish, the gametes of both sexes are activated upon contact with water (freshwater or marine water according to the specific case) and can therefore be maintained in an inactivated state until ready to be experimentally manipulated.

1. Zebrafish *Danio rerio* (Hamilton, 1882)

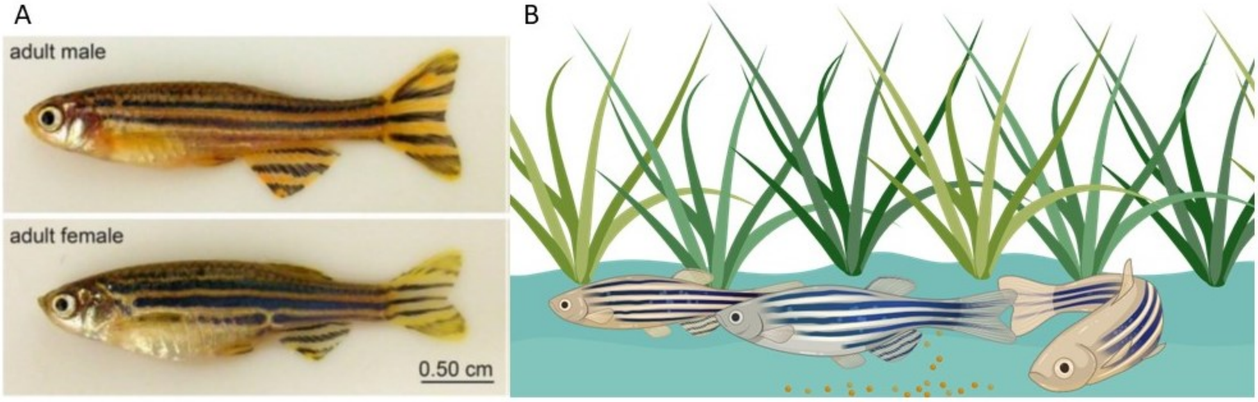

Figure 1. Zebrafish *Danio rerio* A. Adult male and female, showing the sexual dimorphism in body shape and coloration **B**. Illustration of a natural spawning dynamic, where the female is followed in the shallow water by two males during spawning. Made with Biorender.

The zebrafish is a tropical freshwater fish native to the shady, slow flowing water bodies and flood plains of the South-eastern Himalayan region. Zebrafish are a shoaling species and naturally live in mixed sex social groups (Engeszer *et al.,* 2007). Sexual dimorphism is not very pronounced, but it is nonetheless possible to distinguish adult males and females, especially when the latter are ready to spawn. Sexually mature females can be identified by their enlarged belly and prominent genital papilla (Spence & Smith*,* 2006*;* Yossa *et al.,* 2013), while males are usually smaller and slimmer (fig.

1A). Furthermore, females' yellow stripes (particularly in the anal and tail fin) are less saturated compared to those of males, which have a more reddish tone (Spence *et al.*, 2008; fig. 1A).

Zebrafish are group spawners, so pairs usually do not reproduce alone, but in groups of three to seven fish (Spence *et al.,* 2008). In the wild, females ready to spawn rapidly dart multiple times into shallow water (1-2 centimetres deep), chased by one or multiple males (Engeszer *et al.,* 2007; Spence *et al.,* 2008; fig. 1B), often resulting in clutches with multiple paternity (Watt *et al.,* 2011).

Spawning is induced by the light and in nature takes place at dawn (Spence *et al.*, 2007); during spawning, sperm and eggs are released simultaneously and the eggs are scattered into the substrate (Spence *et al.,* 2008). Once released by the female, eggs are activated by contact with freshwater but within 1 minute become non-fertilizable (Yamamoto, 1961).

In nature, zebrafish are an annually breeding species, with the reproductive period starting just before the monsoon time (Spence *et al.,* 2007), but in the lab, with constant temperature and food availability, they breed all year around, with mating intervals ranging from 2 to 8 days (Lawrence, 2007; Yossa *et al.,* 2013). Low maintenance, small size, high fecundity (one female can lay up to 200 eggs a week, Hill *et al.,* 2005), relatively short generation time (in optimal conditions zebrafish can reach sexual maturity in 3 months) and optical transparency during early embryogenesis are some of the characteristics that made zebrafish a popular animal model in a wide range of research fields throughout the years (Lawrence, 2007).

In the context of sexual selection, however, zebrafish has been comparatively little studied, despite its great potential to represent an excellent model for post-mating dynamics. Male competition, indeed, plays a significant role in the reproduction of this species, with matings likely to be determined by the dominant male excluding other males rather than by females actively choosing their mates (Spence & Smith, 2006). In such a scenario, post-mating female control is expected to play a key role in determining the reproductive outcome, thus enabling females to regain some control over the fertilization process. Furthermore, recent findings have shown the potential for FRF to affect post-mating dynamics in this species based on the effects of FRF on sperm traits (Poli *et al.,* 2019), thus opening the possibility for FRF to play a crucial role in the fertilization process.

2. Grass goby *Zosterisessor ophiocephalus* (Pallas, 1814)

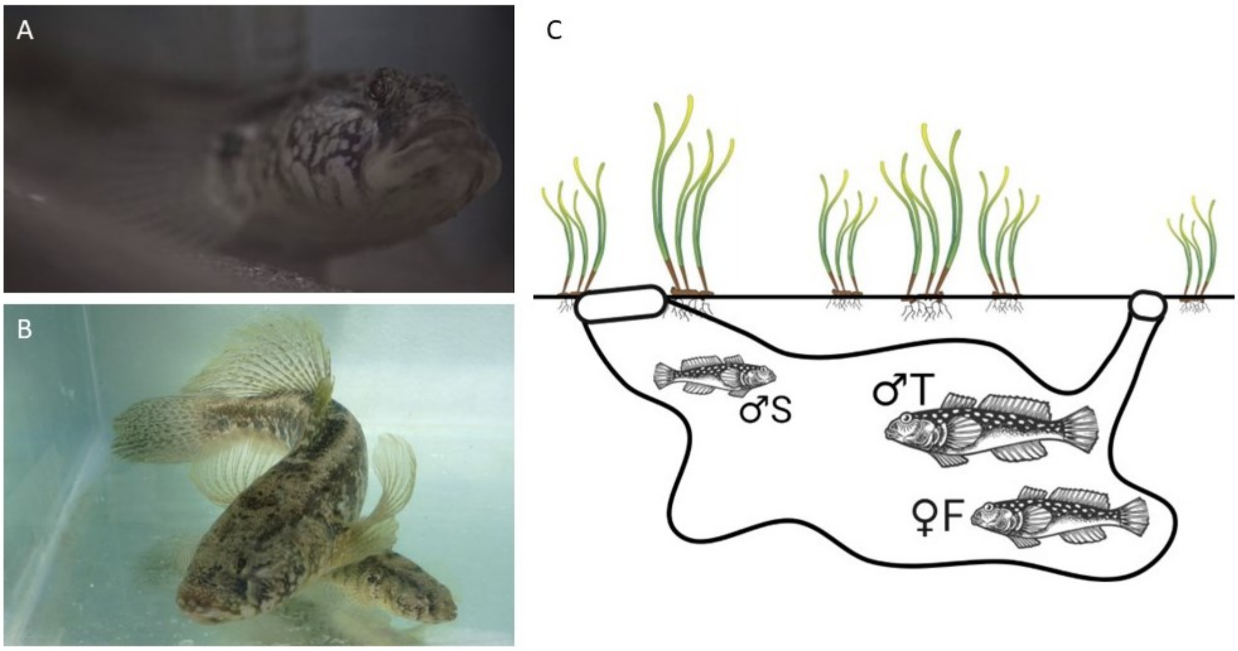

Figure 2. Grass Goby *Zosterisessor ophiocephalus* A. Close up of a territorial male **B**. A territorial and a sneaker male - Sneaker males are smaller compared to territorials and resemble females (the sexual dimorphism is almost non-existent, females are detected from sneakers for the rounder shape of their genital papilla and, during the reproductive season, for their enlarged bellies) Credit: Federica Poli.
C. Schematic illustration of the spawning positions of females, sneakers and territorials inside the territorial's nest. Made with Biorender.

The grass goby is a coastal marine species that inhabits the seagrass meadows of *Zostera spp*. *(Z. nolti* and *Z. marina*) in shallow brackish water (Mazzoldi *et al.,* 2000). Males of this species exhibit alternative reproductive tactics, with larger/older dominant individuals (hereafter: territorials) that, during the breeding season, build and defend a nest (a burrow under the seagrass rhizomes), court females, and provide parental care to eggs. Smaller/younger mature males (hereafter: sneakers), instead, mate opportunistically by sneaking inside territorials' nests and releasing their ejaculates during the spawning event (Scaggiante *et al.,* 1999; Mazzoldi *et al.,* 2000). The territorials' nests usually present one main entrance and one (sometimes two) secondary opening, to enable water flow during fanning and to escape from predators. At the same time, however, the additional openings enable sneaker males to hide from the territorial male until the female starts laying her eggs (Mazzoldi, 2000; fig. 1C).

Territorial males release sperm in the form of mucous trails that slowly dissolves in seawater, thus releasing sperm continuously over a long period of time. They attach these trails on the nest ceiling, very close to where the females lay their eggs one at the time (egg deposition usually lasts several hours) (Marconato *et al.,* 1996; Scaggiante *et al.,* 1999). Sneaker males, on the other hand, are usually kept at a distance from the egg laying females by territorial males and are therefore forced to drop their ejaculates (that in contrast release most of the sperm almost immediately) from an unfavourable position (Mazzoldi *et al.*, 2000; Scaggiante *et al.,* 2005; see figure 1C for a schematic illustration of a territorial's nest and the positions of males and female inside the nest).

This difference in the ejaculates' behaviour reflects the differential allocation of the sperm/seminal fluid proportion of the two alternative tactics, that in turn mirrors the different risk of sperm competition experienced by the males. Sneaker males indeed, that always have to compete with at least one male (the territorial), invest disproportionately more in sperm production and less in seminal fluid, thus maximising their chance of fertilization by releasing all the sperm at once during the spawning event (Scaggiante *et al.,* 1999; Mazzoldi *et al.*, 2000). In contrast, territorials might avoid sperm competition by excluding rival sneakers from the mating event, and to this purpose they show a greater allocation in seminal fluid, allowing to invest time in nest defence while still guaranteeing a constant sperm supply (even though in much lower numbers compared to sneakers) (Scaggiante *et al.,* 1999; Mazzoldi *et al.*, 2000). Despite these differences in sperm and seminal fluid allocation, the two male phenotypes do not differ in sperm velocity and viability (measured both in water and in their own seminal fluid; Locatello *et al.,* 2007). In short, territorial males usually have a proximity advantage (they are preferred by the females and get to lay their sperm trails very close to the egg deposition site), while sneakers have a numerical advantage, producing greater numbers of sperm, that are however released further away from the female.

Because of the intense sperm competition and high frequency of multiple paternity found in the clutches of this species (Mazzoldi *et al*., 2000; Pujolar *et al*., 2012), selection is expected to favour a mechanism allowing directional cryptic female choice (i.e., in favour of specific male phenotypes consistently preferred by the females) to reinforce the pre-mating preference that females show for territorial males. Furthermore, sneaker males of the grass goby can exploit to their advantage the seminal fluid of competitor territorial males, whose sperm performance is in turn penalized by the presence of sneaker seminal fluid (Locatello *et al.,* 2013). Such evolutionary dynamics of ejaculate exploitation and impairment in sperm competition mediated by male seminal fluid makes the grass goby an ideal study system to investigate the combined selective pressures of both sperm competition and cryptic female choice and open the possibility for FRF to play a role in rebalancing the ejaculates competition in favor of territorial males, through an interaction with male seminal fluid.

2. Paper I

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

Female reproductive fluid increases the opportunities for post-mating sexual selection by prolonging egg fertilization window

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ABSTRACT

Female reproductive fluid, the fluid that surrounds the eggs, has attracted increasing attention for its role in fertilization and post-mating sexual selection through its effects on sperm traits. Surprisingly, however, only a few studies have investigated the effects of female reproductive fluid on the eggs. Yet, these effects might offer great potential to affect fertilization dynamics by, for example, increasing the opportunities for post-mating sexual selection. Here, we determined whether, by extending the egg fertilization window (time available for egg fertilization), the female reproductive fluid could also increase the opportunities for multiple paternity. Using the Zebrafish *Danio rerio* we first tested the prediction that female reproductive fluid increases the egg fertilization window, and then, using a split-brood design with sperm of two males added at different times after eggs activation, we tested whether the degree of multiple paternity varies in presence or absence of female reproductive fluid. Our results reveal the potential of the female reproductive fluid to increase multiple paternity throughout its effects on the egg fertilization window thus broadening our knowledge of the mechanisms females in externally fertilizing species affect post-mating sexual selection.

INTRODUCTION

When females mate with multiple males within the same reproductive episode, sexual selection can continue after mating, in the form of post-mating sexual selection (Birkhead & Pizzari, 2002). Traditionally, post-mating sexual selection studies have focused mainly on the interplay among gametes, i.e. sperm and eggs. However, more recently, it has also been demonstrated that the nongametic components released with sperm and eggs have an important role in this stage of sexual selection. Decades of studies on the fluid that surrounds the sperm, namely the seminal fluid, have revealed a multitude of effects that it has on different aspects of the fertilization processes that affect post-mating sexual selection (reviewed in Perry *et al.,* 2013). For example, seminal fluid can deeply affect female remating behaviour and the outcome of sperm competition (i.e. the competition of sperm from two or more males to fertilize the same batch of eggs; Parker, 1970) by affecting sperm competitiveness (Poiani, 2006; Ramm, 2020).

Interestingly, however, in this context, the counterpart of seminal fluid for females seems to have been overlooked. This fluid, recently named (to avoid specific taxa-related terminology) female reproductive fluid ("FRF") (Gasparini *et al.,* 2020) is a fluid that can have different origins (ovarian, oviductal, follicular and/or coelomic, hence explaining some of the different terminology used in the literature such as ovarian fluid, spermathecal fluid, gonoductal fluid, egg water, follicular fluid, etc…) but has the common denominator of surrounding the eggs before and at the time of fertilization. This fluid is kept inside the female reproductive tract in internal fertilizers or is released along with the eggs in external fertilizers, and ultimately is the fluid the sperm come in contact with during their quest to fertilize the eggs (Zadmajid *et al.,* 2019; Gasparini *et al.,* 2020).

The female reproductive fluid has been shown, across a variety of internally and externally fertilizing taxa, to affect multiple sperm traits important for fertilization success, generally enhancing spermatozoa performance. For instance, FRF mediates sperm attraction, prolongs the duration of sperm motility, modulates sperm trajectory, increases sperm viability, and enhances sperm velocity and sperm motility (e.g., Oliveira *et al.,* 1999; Bernasconi *et al.,* 2002; Urbach *et al.,* 2005; Elofsson *et al.,* 2006; Rosengrave *et al.,* 2009; Gasparini *et al.,* 2012; Gasparini and Evans, 2013; Alonzo *et al.,* 2016; Liberti *et al.,* 2016; Poli *et al.,* 2019; Myers *et al.,* 2020).

Interestingly, in recent years, accumulating evidence has revealed that the effects of FRF on sperm traits can extend into post-mating sexual selection, with evidence of FRF as a mediator of cryptic female choice, thus able to differentially affect sperm of different males and ultimately bias the outcome of sperm competition (Firman *et al.,* 2017, Gasparini *et al*., 2020). For example, FRF mediates sperm selection to avoid inbreeding by favouring unrelated males during fertilization in the guppy (*Poecilia reticulata)* (Gasparini & Pilastro, 2011) and the chinook salmon (*Oncorhyinchus tshawytscha*) (Lehnert *et al.*, 2017), while in the external fertilizer mussel (*Mytilus galloprovincialis),* FRF has been shown to attract the sperm of the more genetically compatible males (Oliver & Evans, 2014). Also, FRF has been shown to mediate sperm selection towards the preferred male phenotype, and this seems to occur in species where males show alternative mating tactics, like in the ocellated wrasse (*Symphodus ocellatus),* where FRF decreases the relative importance of sperm number over sperm velocity, thereby penalizing the numerical advantage of sneaker males (Alonzo *et al.,* 2016). Despite this growing body of evidence indicating the role of FRF in post-mating sexual selection processes through its effects on sperm traits, not many studies have investigated the effects of FRF on eggs, and none so far have explored these effects in a perspective of post-mating sexual selection.

The primary role of FRF is to provide the appropriate environment for oocyte maturation, fertilization, and early embryo development (Leese *et al.,* 2001; Aguilar & Reyley, 2005). FRF prolongs egg lifespan in external fertilizers (Dietrich *et al.,* 2012) and improves eggs quality, as it is involved in the protection of eggs from oxidative stress (Agarwal *et al.*, 2005; Da Broi *et al.,* 2018) and pathogens (Johnson *et al.,* 2014). Enzymes of antioxidant defence have been found in the FRF of various species, from insects (Baer *et al.,* 2009) to mammals (Harvey *et al.,* 1995; Fu *et al.,* 2016), and proteomic studies have revealed the presence of proteins related to the immune system in both internally and externally fertilizing species (Seppola *et al.,* 2009; Zamah *et al.,* 2015; Dosselli *et al.,* 2019). Despite this evidence of the effects of FRF on eggs, the consequences of those effects for post-mating sexual selection have yet to be explored.

Here, using the zebrafish (*Danio rerio*) we test for a potential role of FRF in sexual section processes mediated by the effects on eggs rather than on sperm. We asked whether the FRF, by affecting egg viability, might also extend the fertilization window of the eggs (i.e., the time window available for eggs' fertilization) and whether this can translate into increased opportunities for post-mating sexual selection. Indeed, theoretical models (Harts & Kokko, 2013) suggest that the length of the fertilization window might be an important factor able to shift the balance between pre- and post-mating sexual selection, with a wider fertilization window associated with the increased importance of post-mating mechanisms of sexual selection. The zebrafish is well suited to test this hypothesis for many reasons. Zebrafish are group spawners and egg scatterers and, in the wild, females dart repeatedly into shallow water (1-2 cm deep) when ready to spawn, often chased by multiple males (Engeszer *et al.,* 2007; Spence *et al.,* 2008), frequently resulting in broods with multiple paternity (Watt *et al.,* 2011). Once released by the female, eggs are activated by contact with freshwater but within 1 minute become non-fertilizable (Yamamoto, 1961). Recent findings have shown the potential for FRF to affect post-mating dynamics in this species based on the effects of FRF on sperm traits (Poli *et al.*, 2019), thus suggesting also the possibility for FRF to have other effects in the fertilization process.

We first determined the duration of the egg fertilization window and assessed whether the presence of FRF can affect it. Then, using a split-brood design with sperm from two males added at different times from egg activation, we tested the prediction that the presence of FRF can increase the opportunities for multiple paternity, suggesting a novel mechanism of FRF to influence post-mating sexual selection.

MATERIALS AND METHODS

Fish maintenance

Zebrafish used in this experiment were Tuebingen wild type, reared under standard laboratory conditions at the Zebrafish Facility of the Department of Biology (University of Padova, Italy). Adult males and females were kept separated in groups of 15 fish in 3 L tanks in a recirculating rack system (Tecniplast) at a water temperature of 28 ± 1°C with a 12L:12D photoperiod. All fish were fed ad libitum three times per day with a mix of dry food and *Artemia nauplii*. Both males and females used for the experiments were 7-9 months old. All experiments were performed in accordance with the relevant Italian and European Legislations and were approved by the Ethics Committee of the University of Padova (approval number: 100/2019).

Experimental design

Experiment A. Estimating zebrafish fertilization window

We conducted a preliminary experiment to estimate the egg fertilization window in zebrafish in standard conditions (i.e. with no manipulation of the FRF surrounding the eggs). Previous work indicates that eggs can be fertilized up to 60 seconds from activation (that occurs once eggs come in contact with freshwater; Yamamoto, 1961). We tested the length of the fertilization window by adding freshly activated sperm (to avoid the confounding effect of post-ejaculatory sperm ageing) of the same male to 4 different experimentally split egg pools from the same female after 0s, 15s, 30s and 45s from eggs activation. We used 12 females and 12 males in total.

Experiment B. FRF effect on fertilization window

We tested whether the presence of FRF affects the egg fertilization window by comparing the fertilization rate in the presence and absence of FRF at two time points: at 0 and 45 seconds from eggs activation. For each female, eggs were collected, rinsed to remove the original FRF (see “Gametes and FRF collection” section for more details), and split into four equal pools. FRF was then re-added to two of these egg pools. Freshly activated sperm were added to the eggs 0 and 45 seconds after egg activation, and the fertilization success was recorded. For this experiment, we obtained 20 experimental replicates (20 male-female pairs).

Experiment C. Multiple paternity in presence or absence of FRF

We tested whether the effect of FRF on the fertilization window provides more opportunities for post-mating sexual selection by increasing the degree of multiple paternity. To do so, for each replicate we collected ejaculates from two males (labelled A and B) and the eggs and FRF from one female, so each replicate involved two males and one female, for a total of 15 replicates (2 males-1 female triplets). Once collected, the eggs were rinsed (as described below) to remove the FRF and split into two pools with the same number of eggs. In one of the two pools, the FRF was re-added to the eggs. Freshly activated sperm from the first male (male A) were added immediately after egg activation (time 0), and freshly activated sperm from the second male (male B) added after 30 s (time 30s). We took care to use the same amount of sperm from the two competing males. Fin clips from the caudal fin of all the adults in the triplets were taken after gametes collection and preserved in absolute ethanol until used for molecular analyses. Embryos were collected and preserved in absolute ethanol at 30 hours post fertilization (hpf) for paternity analysis.

Gametes and FRF collection

The evening before the experiment, experimental fish were transferred into breeding tanks (1 L), where males and females remained separated by a transparent divider that allowed visual and olfactory contact but prevented physical interaction and spawning. Gametes were collected the next morning following Alavioon *et al.* (2017). In short, the fish were anaesthetized in a solution of MS222 (tricaine methanesulfonate, Sigma Aldrich; 0.17 g/L), gently rinsed with water, and carefully dried in the abdominal and genital area (to prevent accidental activation of gametes by water). Each fish was then placed under a dissecting microscope for the collection of gametes. Males were gently squeezed to release the ejaculate that was collected in a glass micro-capillary, and then diluted in 40 µL of Hank's balanced salt solution (HBSS; Jing *et al.* 2009) and maintained in ice until used (within 1 hour). Females were gently squeezed in the abdominal area to release eggs, along with the FRF, on a glass slide. The FRF was carefully collected with a Drummond micropipette (see Poli *et al.*, 2019) and maintained in ice until use. The eggs were then rinsed of the remaining fluid with a 0.5% solution of Bovine Serum Albumin (pH 8), which allows maintaining eggs in an inactivated state for up to two hours (Sakai, 1997). Both eggs and FRF were always used within an hour from collection.

In vitro fertilization (IVF)

The eggs of each female were divided into four (exp B) or two (exp C) pools (egg number range per pool: 30-60, with the same number of eggs used in each pool from the same female), and then activated with freshwater (FRF-absent treatment, hereafter referred to as ‘no FRF’ treatment) or with FRF + freshwater (at a concentration of 1:10; FRF-present treatment, hereafter referred to as ‘FRF’ treatment). Sperm number was standardized by assessing sperm number with a LUNA™ Automated Cell Counter and diluting each ejaculate accordingly with the Hank’s solution (Cattelan & Gasparini, 2021). In all experiments, sperm were activated with freshwater (1:5 dilution) and added immediately to the appropriate pool of egg. In experiment A sperm were added at 0, 15, 30 or 45 seconds after eggs activation, in experiment B at 0 or 45 seconds from eggs activation, and in experiment C at 0 or 30 seconds from eggs activation. After fertilization, eggs were incubated at 28 °C and checked at 7 hpf to assess fertilization success. The repeatability of the estimation of the fertilization success was confirmed in a separate experiment using 10 pairs of male-female, each one with two replicates at 0s and 30s.

Microsatellite and Parentage Analysis

Tissues for DNA analyses (the whole body of the embryos and fin clips from adults) were preserved in absolute ethanol until required. Genomic DNA was extracted using a protocol for the isolation of PCR-ready genomic DNA from zebrafish tissues (Meeker *et al.,* 2007). All individuals were genotyped at five microsatellite loci (GenBank accession numbers: Z4830, Z20450, Z11496, Z9230, Z1233) in multiplex PCRs performed in 15 µL reaction volumes following a cycling protocol with an initial denaturation step at 95°C for 10 min, 35 cycles of 30 s denaturation at 95°C, annealing at 58°C for 35 s, extension at 72°C for 50 s, and a final extension at 72°C for 30 min. PCR amplifications were performed on a SimpliAmp Thermal Cycler (Applied Biosystems, CA, USA). Amplified fragments were separated by electrophoresis on an ABI 3100 genetic analyzer (ABI PRISM, Applied Biosystems), using the GeneScan™-500 LIZ (Applied Biosystems) as size standard (https://www.bmrgenomics.it). Microsatellites were scored using the software Geneious 8.1.9 (https://www.geneious.com) and paternity was assigned using Cervus 3.0 (Kalinowski *et al.,* 2007) with 95% strict confidence.

Statistical analyses

All statistical analyses were conducted in R v. 3.6.3 (R Core Team, 2020). Repeatability was tested using the “rptR” package with Proportion distribution, based on 1000 permutations. Repeatability of fertilization success was high both at 0 s ($R = 0.024$, CI: 0.003, 0.054, $p < 0.001$) and at 30 s ($R = 0.063$, CI: 0.021, 0.098, $p < 0.001$).

To investigate the effect of FRF on the proportion of fertilized eggs (experiment B), and on the degree of multiple paternity (experiment C), we used a generalized linear mixed effect model ("glmer" function of the "lme4" package) assuming a binomial error distribution. In the first model, the number of fertilized and non-fertilized eggs was added as the dependent variable (using the function "cbind"), while in the second model, the number of eggs sired by the second male (male B) and those sired by the first male (male A) were added as dependent variables (using the "cbind" function). In both models, treatment (presence/absence of FRF) was included as fixed factor and female ID as a random factor to account for the non-independence of the data. No overdispersion was found in both models (assessed using the function "testDispersion" of the package "DHARMa"). The associated P-value of the fixed factors was assessed using the Anova function (type II sums of squares) from the package "car". Model assumptions were checked by inspection of residuals' distribution, using the package "DHARMa".

Averages are presented with their associated SE.

RESULTS

Experiment A. Estimating zebrafish fertilization window

The average percentage of successfully fertilized eggs was: 87.1% ± 1.4% at 0 s, 79.6% ± 1.5% at 15 s, 53.7% ± 4.4% at 30 s and 19.7% ± 4% at 45 s (Fig. 1).

Experiment B. FRF effect on fertilization window

At 0 s, the average percentage of successfully fertilized eggs was 83.7% ± 1.2% (N=20) in the FRF treatment and 80.7% ± 1.2% (N=20) in the no FRF treatment. At 45 s, 25.1% ± 2.5% (N=20) of the eggs were fertilized in the FRF treatment, while the percentage dropped to 16.5% ± 2.1% (N=20) in the no FRF treatment (Fig. 2). The effect of the FRF on fertilization rate was nonsignificant at 0 s ($\chi^2_1 = 3.487$, $p = 0.062$), but was highly significant at 45 s ($\chi^2_1 = 23.557$, $p < 0.001$) (Fig 2).

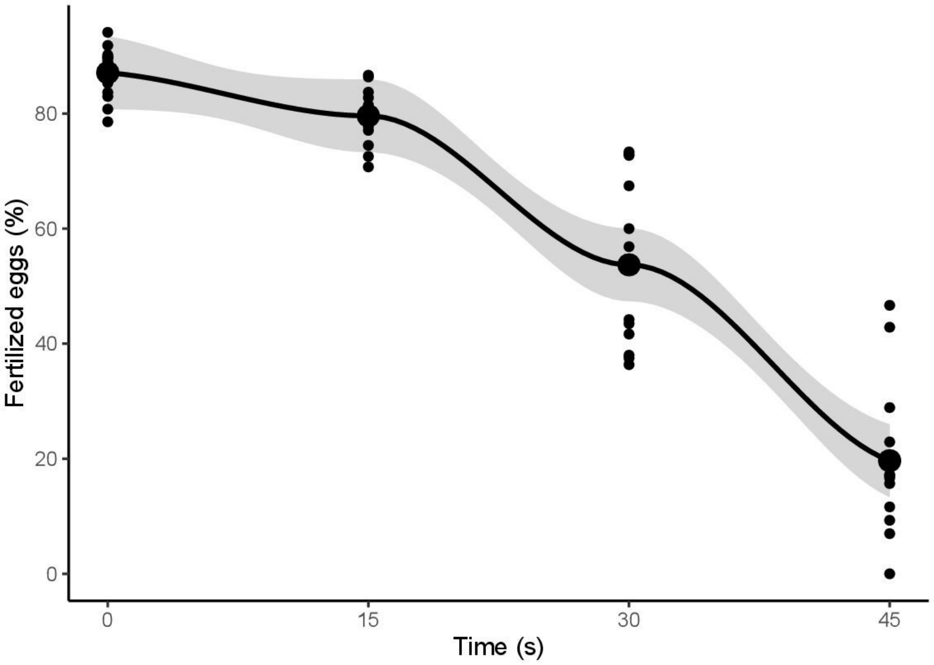

Figure 1. Experiment A. Percentage of fertilized eggs over time obtained from IVFs in standard conditions (i.e., with no FRF manipulation). The individual data points (small dots, N = 12), means (big dots) and standard error of the mean (S.E. shading, in grey) are presented.

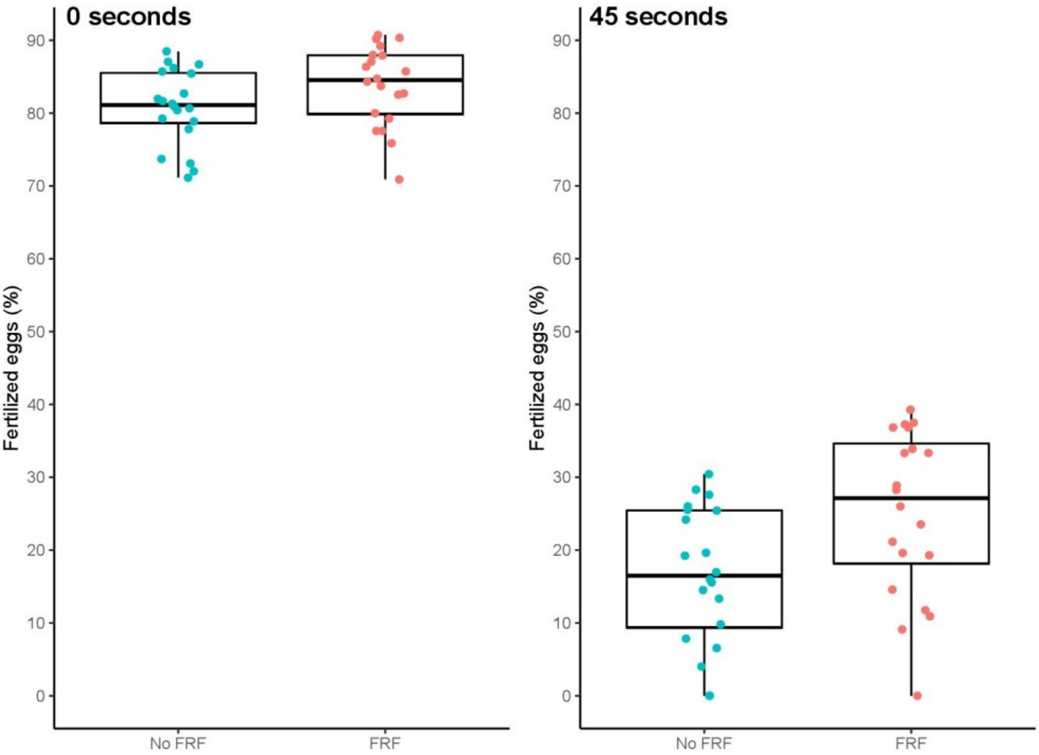

Figure 2. Experiment B. Percentage of fertilized eggs obtained from IVF in absence (blue/left) and presence (red/right) of FRF at 0 s and 45 s. The presence of FRF significantly increased the % of fertilized eggs after 45 s from egg activation. The median (box midline), first (lower box line) and third (upper box line) quartiles, and range (whiskers) are presented. Each individual data point represents an experimental replicate, N=20.

Experiment C. Multiple paternity in presence or absence of FRF

Overall, fertilization success obtained in the FRF treatment was significantly higher than in the no FRF treatment (paired *t*-test: *t*=3.938, df=14, p=0.001, within-pair mean difference of 9.1%). A mean of 51.3% ± 3.2% eggs was fertilized in the FRF treatment and 42.2% ± 3.2% eggs were fertilized in the no FRF treatment. We obtained a total of 541 embryos from 15 triplets (15 females and 30 males). We were able to assign paternity with 95% confidence using CERVUS to 507 (95 %) embryos in total. The paternity was calculated on an average of 16.9 ± 1.3 embryos (range: 6-31) for each group. The second male's paternity (male B) ranged from 0 to 33% (mean: 13.3% ± 2.9%) in the no FRF treatment, and from 11 to 67% (mean: 41.8% ± 4.1%) in the FRF treatment, with an average within-pair difference of 27.7% ± 4%. There was a significant effect of FRF on the relative paternity of the second male ($\chi^2_1 = 41.49$, $p < 0.001$), with the second male, male B, siring more embryos when FRF was present (Fig 3).

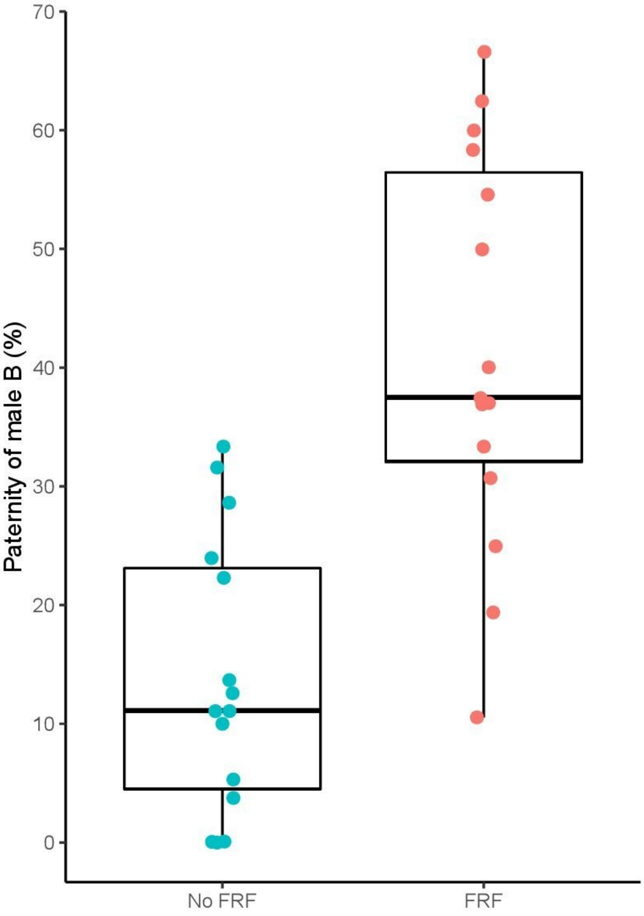

Figure 3. Experiment C. Percentage of paternity obtained by the second male in IVF trials in absence (blue/left) and presence (red/right) of FRF. In presence of FRF, the second male (whose sperm were added 30s after the first male) fertilised significantly more eggs relative to when no FRF was present. The median (box midline), first (lower box line) and third (upper box line) quartiles, and range (whiskers) are presented. Each individual data point represents an experimental replicate, N=15.

DISCUSSION

Overall, our combined results provide evidence that FRF prolongs the time available for egg fertilization (egg fertilization window) in the zebrafish. Moreover, we demonstrated that the effect of FRF on eggs also increases the opportunities for post-mating sexual selection and, as a consequence, the degree of multiple paternity of the offspring. This is the first experimental evidence of a link between the egg fertilization window and the opportunities for post-mating sexual selection in external fertilizers.

We found that the fertilization window of zebrafish's eggs is characterized by a relatively short time frame (at 45 seconds after eggs activation only about 20% of the eggs are still fertilizable), with the majority of the eggs fertilized immediately soon after activation (eggs are activated when they come in contact with water). This result is in line with the timeframe of sperm longevity in this species, where the average duration of sperm motility is often less than 1 min (Wilson-Leedy *et al.,* 2007, Poli *et al.,* 2019). Interestingly, the presence of FRF can prolong both sperm longevity (Poli *et al.,* 2019) and, as we demonstrated in this study, egg fertilization window, thus increasing the possibilities of successful fertilization. The proportion of eggs fertilized towards the end of the fertilization window (45 seconds after eggs activation) increased from 16% without FRF to 25% with FRF. This finding confirms the important role of FRF in maximizing fertilization success for in vitro fertilization protocols in fish husbandry (see, e.g., Turner and Montgomerie, 2002; Lehnert *et al.,* 2017). Mechanisms at the basis of this effect are to be find within the composition of the FRF, as, for example, in zebrafish the FRF contain protease inhibitors that prevent eggs activation (Minin & Ozerova, 2015), and that can play a role also in preserving egg fertilization ability. Furthermore, a selection for longer-lived sperm was previously hypothesized for zebrafish (Poli *et al.,* 2109), since this specific sperm phenotype was shown to sire offspring with higher survival and adult fitness (Alavioon *et al.,* 2019). Therefore, the extension of the egg fertilization window mediated by the FRF could represent a mechanism to reinforce this selection and ultimately increase offspring fitness, but specific studies are needed to test this idea.

Using molecular assignment of paternity, we found that in presence of FRF there was a higher proportion of eggs fertilized by the second male. We estimated paternity on an average of 17 embryos per egg pools, and this was a consequence of our chosen experimental design, and we acknowledge this may have limited the accuracy of paternity estimation in those egg pools with fewer embryos genotyped. However, the use of a paired, balanced design mostly overcome this limitation, but aiming at genotyping more embryos in future studies of this type should be considered for a more precise estimation of paternity. Specifically, in presence of FRF the second male obtained an average proportion of paternity share that was 28% higher compared to IVF in freshwater alone (41% in presence of FRF, 14% in absence of FRF). Our analyses revealed that the increased proportion of the second male is not merely due to the second male fertilizing some of the 'remaining' eggs (those not fertilized by the first male), but to the second male actively competing for fertilization with the first male. Indeed, in presence of FRF there was an overall higher fertilization rate (average difference: 9.1%, min difference: 1%, max difference 31%), but the proportion of eggs fertilized by the second male was larger in magnitude than the cumulative fertilization (average difference: 27.7%, min difference: 4%, max difference 58%), suggesting that the second male fertilize more than the eggs left unfertilised from the first male. Even if further studies are needed to confirm this point, is therefore likely that the second male 'steal' some eggs from the first male fertilization potential.

Therefore, a longer fertilization window provides the opportunity for externally fertilizing females to mate polyandrously, and thus to increase the occurrence and degree of multiple paternity. The evolutionary implications are various, as are the benefits associated with polyandry in the classical (pre-mating) sense. First, there are fertility benefits for females. Indeed, females might benefit from exposing their eggs to multiple ejaculates to ensure an adequate sperm supply to fertilize all the eggs and avoid sperm limitation, thus ultimately enhancing their fecundity (Jennions & Petrie, 2000; Kraus *et al.,* 2004; Snook, 2014). This might represent an insurance against the first male being sterile or releasing few or low-quality sperm. This may be particularly important in species where males become sperm depleted among successive mating events, or when males modulate their sperm investment among different females (Wedell *et al.,* 2002; Birkhead & Moller, 1998).

Moreover, the effect of FRF on multiple paternity has important implications for sexual selection, as it creates more opportunities for post-mating sexual selection to act, and to provide indirect benefits for the females. Multiple paternity in the offspring could be favoured by genetic bet-hedging: the production of more genetically diverse offspring could be a strategy to increase the chances that some offspring will survive in heterogeneous environments characterized by variable selection on fitness-enhancing traits (Jennions & Petrie, 2000). Alternatively, it may be a way for females to bias fertilization towards some specific males and thus exert cryptic female choice, for example to bias fertilization towards unrelated or more compatible partners, or more generally for preferred phenotypes (Eberhard, 1996; Firman *et al.,* 2017). This may be especially important when pre-mating cues are not available or less reliable (Zeh & Zeh, 1997; Birkhead & Pizzari, 2002). In zebrafish, male pre-mating competition plays a significant role in reproduction, with matings likely to be skewed towards the dominant male that is often able to exclude other males from the reproductive event, rather than by females actively choosing their mates (Spence *et al.,* 2008). However, despite this ability of the dominant male, reproduction in zebrafish is often characterized by multiple paternity shared between the dominant and the subordinate male, or males (Watt *et* *al.,* 2011), suggesting that post mating sexual selection plays an important role in the reproduction of this species. Our experimental design mimics this natural situation in which a female spawns in the presence of a male (likely the dominant) and a second male joins the pair slightly later (in our design 30s later); the presence of FRF provides the second male with the opportunity to compete for fertilization and the female with the possibility of exerting a post-mating mate choice.

In conclusion, our findings corroborate and expand the role that FRF plays in post-mating sexual selection, adding new information on how females of externally fertilizing species may affect these processes. It is precisely in these species, where females have limited control over males' competition for fertilization, that post-mating mechanisms are expected to play a key role (Evans & Sherman, 2013). Nonetheless, known mechanisms of cryptic female choice in external fertilizers are limited compared to internal fertilizers, and those mediated by the FRF seem to be one of the best candidates (see Firman *et al.,* 2017). However, little is known about how FRF can affect competitive fertilization (Gasparini *et al.,* 2020), so that the findings of this study expand the horizons of how FRF can affect sexual selection processes, by adding a new mechanism acting on the eggs rather than on the sperm that creates novel opportunities for sperm competition and multiple paternity in external fertilizers.

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Statement of authorship

P.L., P.F., R.M.B. and G.C. conceived the study, P.L. and G.C. designed the experiments. P.L. developed the methods, collected and analysed the data and wrote the original draft. G.A. contributed to the development of paternity methods. G. C. acquired the funding and participated in data analysis. All authors reviewed and edited the draft and contributed to the final version of the manuscript.

Data Accessibility

All data files are available from the Dryad depository https://doi.org/10.5061/dryad.83bk3j9tr

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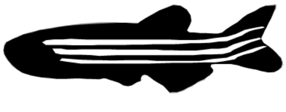
3. Paper II

In preparation for submission to a peer-reviewed journal.

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



Sperm velocity in the race for fertilization – the influence of female reproductive fluid

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ABSTRACT

Accumulating evidence has demonstrated that often sperm number and performance alone cannot predict the outcome of sperm competition, as the female reproductive environment can interfere with the process. Female reproductive fluid (FRF) has recently been proven to differentially affect the sperm performance of different males and bias fertilization towards specific partners. Yet, whether and to what extent the effects of FRF on sperm traits might proportionally translate into paternity biases, and predict the outcome of sperm competition, is still unknown. Here, using the zebrafish *Danio rerio*, we compare the paternity share and predictors of fertilization success of two competing males in presence and absence of FRF, using in vitro fertilization with a split batch design and assessment of sperm performance within the same experiment. Our results reveal the direct association in direction and magnitude of the FRF effects on sperm performance and on the paternity share of male competitors, while also characterizing predictors of sperm competitiveness in the zebrafish for the first time. Specifically, the change in sperm velocity caused by FRF was found to directly predict the change in paternity share between rival males from water to FRF, providing valuable insights into the mechanisms by which FRF can influence fertilization.

INTRODUCTION

Polyandry (i.e., female promiscuity) is a taxonomically widespread phenomenon (Birkhead & Moller, 1998; Simmons, 2001; Griffith *et al*., 2002), with evidence across taxa demonstrating that females actively seek copulations with multiple partners within the same reproductive cycle (Birkhead, 2000). The evolutionary implication of polyandry is the possibility for sexual selection to extend after the mating event (Parker, 1984; Tregenza & Wedell, 2000). Sperm competition, i.e., the competition among ejaculates from different males to fertilize the same set of eggs, has been associated with the rapid evolution and diversification of male traits enabling ejaculates to outcompete those of rivals. Those traits span from the modulation of sperm production and allocation to the fine tuning of sperm quality traits, such as sperm velocity, motility, viability and/or longevity (Snook, 2005; Immler *et al.*, 2010; Parker & Pizzari, 2010; Simmons & Fitzpatrick, 2012; Evans *et al.,* 2013). Despite the increasing knowledge of the proximate mechanisms involved in sperm competition, making predictions on its outcome often remains challenging and far from straightforward. It has indeed become clear that most of the time the paternity share among competing males does not depend only on the relative differences in their sperm number or performance. An important factor that may shuffle the cards on the table has often been overlooked: the female reproductive environment. The arena in which sperm competition occurs is indeed largely shaped by the females, with their reproductive tracts or their derivates allowing females to exert control over sperm competition, a process known as cryptic female choice (Thornhill, 1983; Eberhard, 1996).

An increasingly investigated component of the female reproductive environment, by which females of both internally and externally fertilizing species can influence and control the fertilization process, is the female reproductive fluid. The term female reproductive fluid (hereafter, FRF) refers to any medium, produced by the females, through which sperm must pass on their way to fertilize eggs (Gasparini *et al.,* 2020). This fluid is kept inside the female reproductive tract in the case of internal fertilizers, or released in the external environment, when eggs are externally fertilised.

Recent evidence has identified FRF as a mediator of cryptic female choice, allowing females to differentially affect the sperm of different males and influence the outcome of sperm competition. FRF has been reported to have differential effects on the sperm performance of male competitors in a variety of species (Urbach *et al.,* 2005; Rosengrave *et al.,* 2008; Evans *et al.,* 2013; Evans & Sherman, 2013; Poli *et al.,* 2019) and even in humans (Fitzpatrick *et al.,* 2020). Furthermore, recent studies have shown that FRF is also able to bias paternity, favouring fertilization from unrelated, or genetically compatible, partners, or preferred male phenotypes (Gasparini & Pilastro, 2011; Oliver & Evans, 2014; Alonzo *et al.,* 2016; Makiguchi *et al.,* 2016; Lehnert *et al.*, 2017).

Whether, and to what extent, the direction and magnitude of the phenotypic effects of FRF on sperm performance might directly translate into differences in paternity bias, and hence predict the outcome of sperm competition, has however yet to be investigated. Indeed, previous research has demonstrated that FRF plays an important role in determining shifts in paternity biases that would otherwise be attributed solely to sperm traits of the competing males, but the actual magnitude of this effect is not yet understood. To understand the strength of the selection imposed by FRF at the post-mating stage it is first necessary to rule out the contribution of pre-mating selective episodes, and for this purpose an externally fertilizing study system is ideal, as in vitro fertilization neatly allows to remove all male-female interactions prior to fertilization, to narrow down the contribution of post-mating selective forces. Furthermore, to disentangle the different male and female components contributing to the paternity success, it is necessary to control for female and genetic compatibility effects, and to accurately assess male post-mating traits in presence and absence of FRF concomitantly with the fertilization event. We were able to do so, and account for all these factors, by using the zebrafish *Danio rerio* as model species, building on previous knowledge of the FRF effects on sperm traits (Poli *et al.,* 2019). Additionally, the use of a split-clutch design enabled us to control for intrinsic male-female effects due the random assignment of competitors males and female in the sperm competition assays.

The zebrafish is being increasingly employed as a suitable model for post-mating dynamics. In the wild, females ready to spawn rapidly dart multiple times into shallow water (1-2 cm deep), chased by one or more males (Engeszer *et al.*, 2007; Spence *et al.*, 2008), frequently leading to clutches with multiple paternity (Watt *et al.*, 2011). Matings are often determined by the dominant males excluding subordinates, making post-mating female control crucial, as it could enable females to regain some control over the fertilization process. (Spence & Smith, 2006)

Indeed, recent findings in this species have confirmed that FRF can affect post-mating dynamics, through a differential modulation of the sperm performance of different males (Poli *et al.,* 2019) and through an increase in the opportunities for post-mating sexual selection mediated by a prolongation of the egg fertilization window (Pinzoni *et al.,* 2023).

The overall aim of this study is to unravel the strength and direction of FRF effects on the paternity share among different males and to relate this fitness effect with the strength and direction of the phenotypic effects of FRF on sperm performance traits. Specifically, we were interested in studying whether the direction and magnitude of the paternity shift in presence or absence of FRF can be predicted by the change in sperm traits caused by FRF. To do so, we compared the paternity share and the predictors of fertilization success between two competing males in presence and absence of FRF. We employed an experimental approach based on in vitro fertilization (IVF) with a split batch design, in which fertilization of the same set of eggs took place with or without FRF, and where predictors of fertilization success (sperm motility, swimming velocity, trajectory, and longevity) were assessed in presence and absence of FRF, all within the same experiment. This also allowed us to identify the predictors of competitive fertilization outcome both in standard conditions - no FRF - and with FRF, in the zebrafish, which has never been studied before.

MATERIALS AND METHODS

Fish maintenance

Zebrafish used in this study were Tuebingen wild type, reared under standard laboratory conditions at the Zebrafish Facility of the Department of Biology (University of Padova, Italy). All fish were kept in mixed sex groups of 15 fish in 3 L tanks in a recirculating rack system (Tecniplast) at a water temperature of 28 ± 1°C with a 12L:12D photoperiod and fed ad libitum three times per day with a mix of dry food and *Artemia nauplii*. Adult males and females were allowed to spawn (in groups of 6-8 fish with balanced sex ratio) every week to preserve their optimal reproductive health. For a week prior to the experimental procedure males and females were kept separated with a barrier that allowed both olfactory and visual stimuli. Both males and females used for the experiments were 9-11 months old. All experiments were performed in accordance with the relevant Italian and European Legislations and were approved by the Ethics Committee of the University of Padova (approval number: 100/2019).

Overview of the experimental design

We tested whether the presence of FRF affects the outcome of sperm competition by comparing the fertilization success of two competing males in presence and absence of FRF and measuring sperm performance of the two males in presence and absence of FRF. To do so, for each replicate we collected the eggs and the FRF from one female and the ejaculates from two males (randomly labelled A and B), for a total of 20 replicates (unique triplets of 2 males and 1 female). After collecting FRF from the eggs, these were rinsed (for more details see the section “Gametes and FRF collection”) and split into two pools with equal number of eggs. In one of the two pools the FRF was re-added before the IVF (FRF treatment) while not in the other (NO FRF treatment). The same number of sperm from the two males A and B were mixed in an Eppendorf tube, activated with water, and immediately added to the eggs. At the same time, the sperm performance of the two males was assessed both in freshwater and in the FRF used for the IVFs.

Photographs of anaesthetised males were taken to measure body size and coloration.

Fin clips from the caudal fin of all adults were collected and preserved in absolute ethanol for the subsequent molecular analyses. Embryos were collected and preserved in absolute ethanol at 30 hours post fertilization (hpf) for paternity analysis.

Gametes and FRF collection

Gametes were collected in the morning from fish kept in the dark, as egg deposition in this species is stimulated by the first light (Spence *et al.,* 2008), following Pinzoni *et al.,* 2023. Briefly, the fish were anaesthetized in a solution of MS222 (tricaine methanesulfonate, Sigma Aldrich; 0.17 g/L), rinsed with water, and carefully dried in the abdominal and genital area to prevent accidental activation of gametes by water. Each fish was then placed under a dissecting microscope for the collection of gametes. Males were gently squeezed to release the ejaculate that was collected in a glass micro-capillary, diluted in Hank's solution (HBSS; Jing *et al.* 2009) and maintained in ice until used (within an hour from collection). The number of sperm from each male was counted (with a LUNA™ Automated Cell Counter) and diluted accordingly to equalise sperm number between males for subsequent use for IVFs and CASA analysis. Females were gently squeezed in the abdominal area to release eggs along with the FRF on a glass slide. The FRF was carefully collected with a Drummond micropipette (see Poli *et al.*, 2019) and maintained in ice until use (within an hour from collection). The eggs were then rinsed with a 0.5% solution of Bovine Serum Albumin (pH 8), which allows maintaining eggs in an inactivated state without compromising egg quality (Sakai, 1997).

In vitro fertilization (IVF)

IVFs were performed following Pinzoni *et al.* (2023). In short, the eggs of each female were divided into two pools (average egg number per pool: 33.2 ± 1.3) with the same number of eggs used in each pool from the same female), and then activated with freshwater in the FRF-absent treatment (hereafter referred to as 'no FRF' treatment) or with FRF + freshwater (final concentration of FRF: 10%) in the FRF-present treatment (hereafter referred to as 'FRF' treatment). Equal amount of sperm from the two competing males in the triplet were mixed in the same tube, activated with freshwater (1:5 sperm-water proportion) and added immediately to the eggs. After fertilization, eggs were incubated at 28 °C and checked at 7 hpf to assess fertilization success. We obtained a total of 21.7 ± 1.2 fertilised eggs for each treatment level.

Analysis of sperm performance

For each assay, 0.5 µL of ejaculate were transferred into a chamber on a 4-chambers slide (Leja) and activated in 2.5 µL of freshwater or of the FRF solution in randomized order. Sperm quality from each male were assessed in both conditions. Sperm performance was assessed using a CEROS Sperm Tracker (Hamilton-Thorne Research, Beverly, MA), from which we obtained the following parameters: sperm average path velocity (VAP), straight Line Velocity (VSL), curvilinear velocity (VCL), linearity of the sperm trajectory (LIN, measure of path curvature), and sperm motility (proportion of motile cells over the total). For each sample, we assessed with the sperm tracker an average of 299.6 ± 7.1 SE sperm cell tracks. Sperm longevity was measured as the time from activation until ≥80% of sperm in the field of view were immotile (Poli *et al.,* 2019).

Microsatellite and Parentage Analysis

We performed paternity analysis on a total of 60 adults (40 males and 20 females) and 808 embryos (average embryos number per treatment group: 20.1 ± 1.1). Genomic DNA was extracted using a protocol for the isolation of PCR-ready genomic DNA from zebrafish tissues (Meeker *et al.* 2007). All individuals were genotyped at five microsatellite loci (GenBank accession numbers: Z4830, Z20450, Z11496, Z9230, Z1233) in multiplex PCRs performed following Pinzoni *et al.* (2023). The amplified fragments were separated by electrophoresis on an ABI 3100 genetic analyzer (ABI PRISM, Applied Biosystems), using the GeneScan™-500 LIZ (Applied Biosystems) as size standard (https://www.bmrgenomics.it). Microsatellites were scored using the software Geneious 8.1.9 (https://www.geneious.com) and paternity was assigned using the software Cervus 3.0 (Kalinowski *et al.,* 2007) with 95% strict confidence. We were able to assign paternity to 803 out of the 808 analysed (97%).

Colour analysis

Photographs of males were taken using a Canon Esos 450D, with a millimetric reference and a simulated Gretag Macbeth Pantone in each image to provide a reference for size and colour calibration. We measured body size (body length and area) and spectral properties of blue/violet body stripes and yellow stripes of the anal fin and tail. We measured these colorations to explore possible indicators of sperm traits in males. Carotenoid-based coloration (in the zebrafish the yellow coloration on the tail and fins) is widespread in fish and other animals, and often correlate with sperm quality (e.g. the three-spined stickleback – Mehlis *et al.*, 2013). We also considered the blue/violet coloration of the stripes on the abdomen as recent evidence suggests that this coloration may be associated with pre-mating female choice (Santacà & Gasparini, unpublished results).

To estimate the reflectance of male colour stripes from the photographs we used ColourWorker (http://www.colourworker.com). We considered one blue/violet stripe on the abdomen, one yellow stripes from the anal fin and one yellow stripe from the caudal fin.

For all the experimental males, we obtained 61 reflectance measures taken at 5-nm wavelength intervals in the visible light range (between 400 and 700 nm) for each stripe. Then, following Cuthill *et al.* (1999), to reduce the number of measures per colour stripe per individual, we used principal component analysis (PCA), considering only principal components (PCs) with eigenvalues greater than 1 for all the three stripes. We obtained three PCs for the blue/violet stripe and the yellow stripe in the anal fin, and two PCs for the yellow stripe in the tail fin (table S1 supplementary materials). We therefore considered for subsequent analyses only PC1 and 2 (explaining <95% of the total variation for all colour stripes), corresponding, respectively, to the total spectral reflectance (a standard measure of brightness) and the intensity/purity of the specific colour (an equivalent of chroma, a measure of the shape of the reflectance spectrum).

Statistical analyses

Analyses were performed using R v. 4.2.0 (R Core Team, 2022). Paternity share was analysed using a generalised linear mixed effects model (‘glmer’ function of the ‘lme4’ package; Bates *et al.,* 2015) assuming a binomial error distribution. The proportion of offspring sired by male B was fitted as response variable (using cbind function cbind(successes, failures), to combine the number of offspring sired by male B and the offspring sired by male A). As predictors we fitted the relative differences among competing males (male B -male A trait) in sperm traits and with the interaction with treatment to explore differences in predictors of paternity among IVFs conducted in presence or absence of FRF. The models were fitted with family ID and male ID as random factors to account for non-independence of the data. We calculated p values of the fixed effects by Type II Wald chi-square tests using the ‘Anova’ function of the ‘car’ package (Fox & Weisberg, 2019) but when in presence of significant interactions, Anova type 3 was used instead. Overdispersion was checked in binomial models, but there was no need of correction. Finally, to assess whether the change in sperm velocity mediated by the FRF reflects the change in paternity, we built a linear mixed model in which as dependent variable we added the difference in the paternity -proportion of offspring sired- in the FRF minus offspring proportion without FRF, and as factor the difference in sperm velocity measured in the FRF minus sperm velocity measured without FRF (family ID and male ID were added as random as above). Residuals of the models were visually inspected with the QQ plots to check model assumptions. Data are reported as means ± SE.

RESULTS

Correlation among pre and post mating male traits

Comprehensive results of the multivariate correlation analysis can be found in table S2 of the supplementary materials. We evidenced a high positive correlation among the three parameters of sperm velocity obtained from the sperm performance analysis (Average Path Velocity VAP, Straight Line Velocity VSL and Curvilinear Velocity VCL), and hence selected only VCL, for further analysis, as it seems to be the most employed sperm velocity parameter for this species (Wilson-Leedy & Ingermann, 2007; Poli *et al.,* 2019).

A positive correlation was found also between sperm velocity (VAP+VSL+VCL) and both sperm motility and longevity, as well as with the PC2 for the yellow stripe of the anal fin (representing yellow chroma), even though weaker. Furthermore, sperm motility and longevity were found to be positively correlated with each other. Sperm linearity turned out to be negatively correlated to male standard length. Finally, we registered a positive correlation among the PC1s of the three colour stripes, indicating a positive relation between the yellow and blue brightness, and some additional correlations between the PCs 1 and 2 across the different colour stripes (see table S2 for the specific combinations).

Fertilization rate

Fertilization rate was significantly higher in FRF compared to water (proportion of fertilised eggs over the total, FRF: 0.69 ± 0.03, water: 0.61 ± 0.03, z=3.277, P=0.001).

Predictors of paternity share with or without FRF

Results from the mixed model investigating the impact of sperm traits in determining competitive fertilization outcome in water and FRF are reported in table 1. In presence or absence of FRF the main predictor of paternity share was the difference among competing males in sperm velocity (VCL), with males having faster sperm compared to the competitor gaining relatively more paternity. There was no significant interaction between sperm velocity and treatment, indicating that in both FRF and no FRF treatment sperm velocity is a significant predictor of sperm competition (water: r=0.685, P<0.001, FRF: r=0.653, P=0.002, fig 1). There was instead a significant interaction between treatment and sperm longevity, with sperm longevity significantly correlated with paternity in water (r=0.66, P=0.001) but not in FRF (r=0.288, P=0.217).

Table 1. Results from mixed effect model investigating the impact of sperm traits on the outcome of competitive fertilization, and interactions of sperm traits with the experimental treatment (presence/absence FRF).

	Chisq	***Df***	***P***
Difference in sperm velocity between males (B-A)	17.284	1	**<0.001**
Difference in sperm linearity between males (B-A)	0.046	1	0.831
Difference in sperm motility between males (B-A)	0.365	1	0.546
Difference in sperm longevity between males (B-A)	2.242	1	0.134
*Difference in sperm velocity between males * treatment*	2.237	1	0.135
*Difference in sperm linearity between males * treatment*	0.131	1	0.718
*Difference in sperm motility between males * treatment*	1.479	1	0.224
*Difference in sperm longevity between males * treatment*	6.281	1	**0.012**

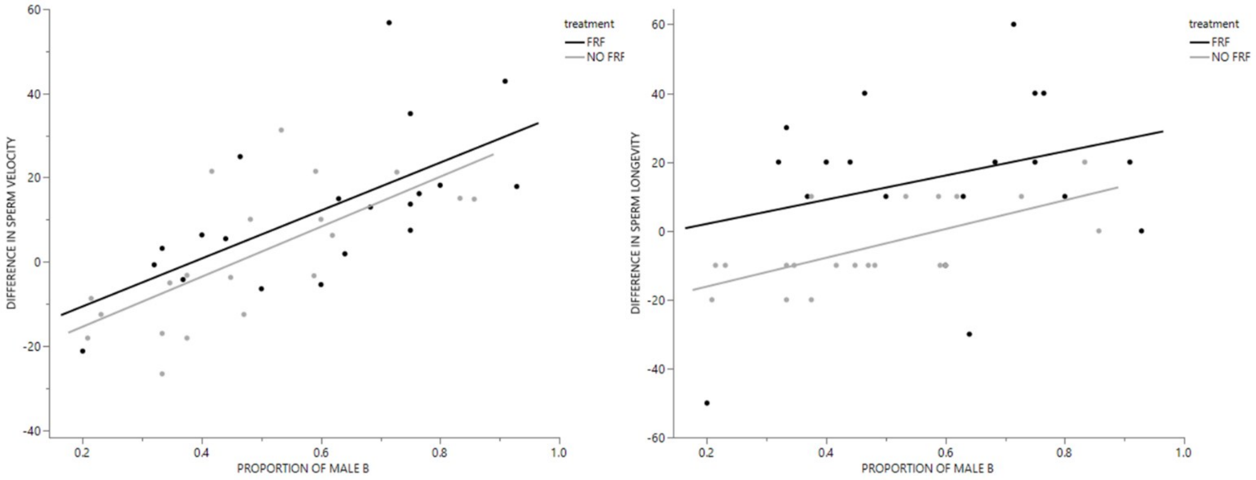

Figure 1. Relationship between the difference in sperm curvilinear velocity (left) and longevity (right) between the two competing males and the proportion of offspring sired by one of the two males (here, randomly, male B). Each individual data point represents an experimental replicate, i.e. a pair of males, N=20, across treatments: in black presence of FRF, in grey absence of FRF.

There was no effect of pre-mating traits (male body size and coloration) on paternity, except for the interaction between treatment and PC2 of the yellow tail (χ^2_1=11.148, P<0.001), with males with more intense yellow negatively associated with lower paternity in water (r=-0.621, P=0.0034), but not in FRF (r=0.118, P=0.6192).

Linking FRF effect on sperm to paternity change

As expected, FRF on average increased sperm velocity compared to water (paired t-test: t_{39} = 3.1745, p=0.002) of 6.8 ± 2.15 (in percentage an increase of 7.1 % ± 2.07), but with some variation (min: -30.3, max: 39.2).

The variation in sperm velocity from water to FRF, a measure of how much the FRF increased the velocity compared to the intrinsic quality of sperm (measured in water), translated in the variation in the paternity change from IVF with or without FRF (χ^2_1=24.083, P<0.001). In cases where the FRF decreased sperm velocity, paternity of the male in FRF decreased compared to water, when the phenotypic effect of FRF on increasing sperm velocity was large, also the effect was large. Thus, there was a correlation in the direction and strength of FRF phenotypic effect on sperm velocity and the competitive paternity outcome (r=0.626, p<0.001, fig 2).

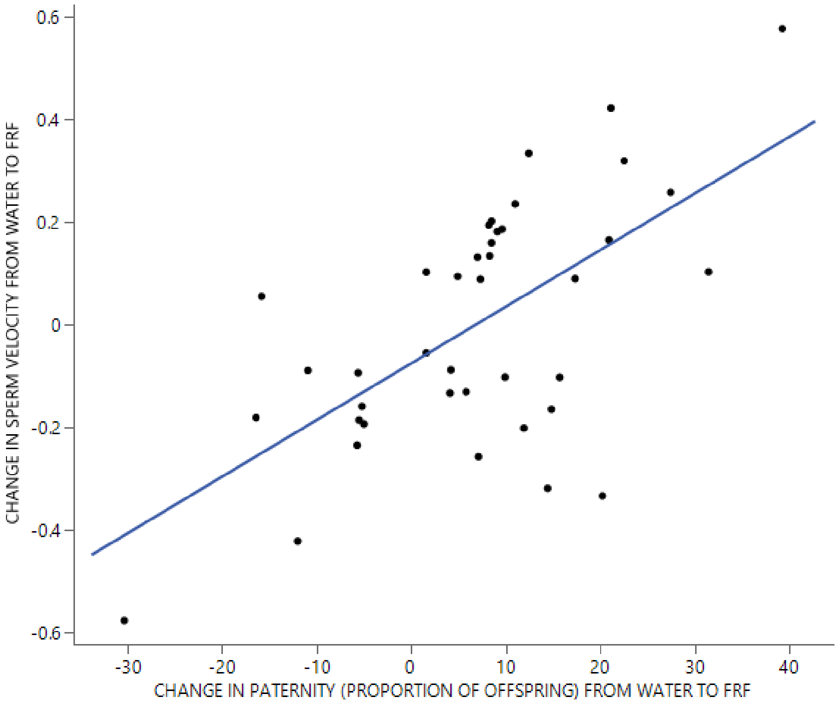

Figure 2. Relationship between the phenotypic effect of FRF on sperm curvilinear velocity (percentage difference in VCL from water to FRF) and the competitive paternity outcome from water to FRF. Each individual data point represents a male (N=40).

DISCUSSION

The findings of this study are multiple, with the most striking being that we reported for the first time the direct association in magnitude and direction between the phenotypic effect of FRF on sperm performance and the fitness effect in terms of increase/decrease in paternity share. Both in absence and presence of FRF, the relative differences in sperm swimming velocity among competing males was the main predictor of paternity share. Interestingly, the influence of FRF on sperm velocity was mainly positive, with different magnitudes depending on the specific male-female combination variation, as previously found in the same species (Poli *et al.,* 2019) but with some cases in which the effect was negative. The variation in sperm performance caused by the FRF was therefore in both directions (from -30 to +39 µm/s). Such a variation in sperm velocity caused by FRF turned out to directly predict the change in paternity share between the competing males caused by FRF. In particular, the magnitude of the change was positively associated with the magnitude of the change in sperm velocity from absence to presence of FRF. This indicates that the change in sperm swimming velocity mediated by the FRF could serve as an indicator of female post-mating preference for specific males, then resulting in a paternity bias towards that male. Our combined results constitute the first evidence of a direct link between the phenotypic effects of FRF on sperm performance and its influence on the paternity share of competing males, effectively showing how FRF influences fertilization dynamics by acting on the main predictor of male post-mating success, sperm swimming velocity.

This study ultimately confirms the pivotal role of FRF in affecting fertilization dynamics at the gametic level, while also revealing the mechanism by which this occurs, through a differential effect on sperm velocity resulting in a fertilization bias. Such a mechanism of cryptic female choice had already been suggested based on accumulating evidence showing that, for example, in the chinook salmon, the difference in sperm velocity of rival males assessed in the presence of FRF is the best predictor of their fertilization success (Rosengrave *et al.,* 2008; Rosengrave *et al.,* 2016). Another study, conducted using the ocellated wrasse, showed how the presence of FRF can change the paternity share among competing males (Alonzo *et al.,* 2016). Our study adds to this previous evidence, but with a twist. Differently from the previous, we focused on sperm performance by standardizing sperm number among males, and we combined in the same experiment sperm velocity measurements and paternity assays both in presence and in absence of FRF within the same triplet of individuals (two males and one females). This has never been looked at the same time, and by doing so we were able to demonstrate the direct association between FRF effects on sperm behaviour and paternity outcome. We therefore were able to compare a situation where presumably only sperm competition operates (absence of FRF), with a competitive dynamic in which sperm competition and cryptic female choice operate simultaneously (presence of FRF), thereby disentangling the male from the female-driven effects.

This is also the first study characterizing predictors of sperm competition success in the zebrafish. Despite being a very well-known model organism, the zebrafish has been not so well explored in the context of sexual selection, and very little is known to date about both pre-and post-mating selective dynamics in this species. Here, we showed that the relative differences in sperm swimming velocity and longevity among rival males are the best predictors of paternity success for the zebrafish, corroborating a long series of studies in fishes demonstrating that sperm velocity is the main determinant of male competitive fertilization success (e.g., Gage *et al.,* 2004; Skjaeraasen *et al.,* 2009; Gasparini *et al.,* 2010; Boschetto *et al.,* 2011).

We also measured some morphological traits in males (including body size, coloration) to check for possible associations between pre- and post-mating traits, but found little evidence of this, at least for the traits we have considered. Also, influence of pre-mating traits on paternity success (perhaps not mediated by the sperm traits we measured) was negligible, whereas the contribution of post-mating sperm traits had a significant impact on the observed variation in paternity success. This finding aligns with the mating system of this species, characterized by a limited female pre-mating control and intense male-male and sperm competition (Spence *et al,* 2008).

Interestingly, in a previous study on the same species, FRF was shown to increase the opportunities for post-mating sexual selection, by acting on the egg fertilization window, ultimately also increasing the opportunities for females to gain control over the fertilization process (Pinzoni *et al.,* 2023). Here, we demonstrated that, by affecting male competitors' sperm behaviour, FRF effectively bias sperm competition outcome, enabling female to exert cryptic female choice via FRF. The combined results of these two experiments point towards a scenario where FRF mediates a complex interplay of multiple effects, working in synergy to ultimately enable females to favour the preferred partner at the post-mating level.

It remains to be determined which characteristics make a male the preferred partner in this species. In other species, FRF was shown to bias fertilization towards unrelated, or genetically compatible, partners (Gasparini & Pilastro, 2011; Oliver & Evans, 2014; Lehnert *et al.*, 2017), identifying FRF as a mediator of non-directional cryptic female choice, where the post-mating preferences of individual females for specific males vary, generating non-directional selection (Firman *et al.,* 2017). Based on our results, together with unpublished results on pre-mating female choice based on genetic compatibility at the MHC loci in the zebrafish (Santacà, Grapputo & Gasparini), it is likely that the cryptic female choice mediated by the FRF in the zebrafish fall into the same category, i.e., compatibility. The change in paternity share detected across treatments (absence/presence of FRF) could indeed be attributed to a FRF-mediated mechanism favouring the most genetically compatible partner. The top candidates for mediating the recognition of the sperm from the most compatible male are genes linked to the major histocompatibility complex (MHC), a group of highly polymorphic genes that play a critical role in the immune function and self/non-self recognition (Milinski, 2022). The MHC genes have emerged as important components of mate choice at the post-mating level, not only in internally fertilizing species, in which selection occurs within the female reproductive tract (for example, in the Red junglefowl *Gallus gallus* - Løvlie *et al.,* 2013 - and in guppy *Poecilia reticulata* - Gasparini *et al.,* 2015), but also in some externally fertilizing fish (e.g., the chinook salmon Neff et al., 2008 and in the Atlantic salmon Yeates *et al.,* 2009).

In conclusion, the mechanism of post-mating female control described here, mediated by FRF through a differential modification of sperm swimming velocity, may be taxonomically widespread, as FRF seems to be a fundamental component of the female reproductive environment, with a high potential for interacting with both gametic and non-gametic male components.

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Statement of authorship

P.L., R.M.B. and G.C. conceived the study, P.L. and G.C. designed the experiments. P.L. developed the methods, collected the data, and wrote the original draft. G.C. acquired the funding and analysed the data. All authors reviewed and edited the draft and contributed to the final version of the manuscript.

SUPPLEMENTARY MATERIALS

Colour analysis - details

The program ColourWorker (http://www.colourworker.com) compares the reference spectra of the specific subject being analysed (here, the zebrafish) to the spectral information obtained from the Gretag Macbeth Pantone present in every picture. In doing so the program can compensate for variations in reflectance resulting from changes in the environmental lighting or in the camera's light encoding capability (Grueber *et al.,* 2017). The reference spectra were obtained from a subsample of 24 males (from our population of Tuebingen wild type) using a USB2000 spectrometer (Ocean Optics Inc., Dunedin, FL) and a Deuterium Tungsten light source (Analytical Instrument Systems Inc., NJ).

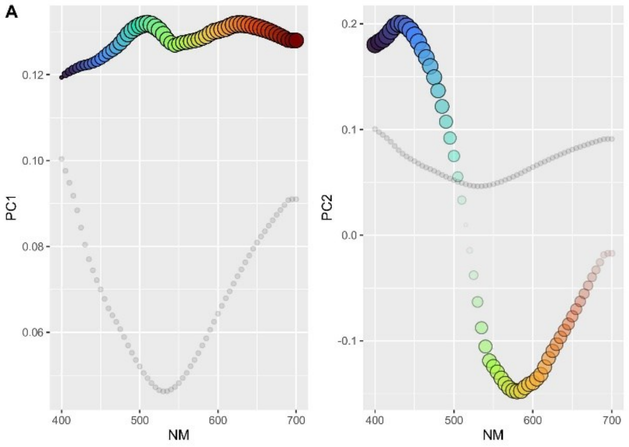
Supplementary Table S1 Eigenvalues and % of variance explained for the principal components obtained for blue/violet body stripe and yellow stripes of tail and anal fin. PCA were performed on the mean spectra obtained for each stripe separately and was conducted on the correlational matrix without rotation (Grill & Rush 2000). Only PCs with eigenvalues greater than one for all the colour stripes were considered in subsequent analyses.

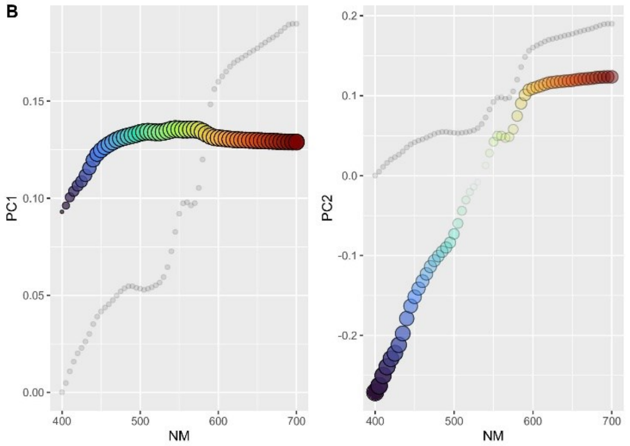
	PCs	eigenvalues	% Variance	Total % Variance
Blue/violet	PC1	52.96	86.82	99.73
	PC2	5.14	8.43	
	PC3	2.73	4.48	
Yellow tail	PC1	53.14	87.11	99.71
	PC2	7.17	11.75	
Yellow anal	PC1	48.26	79.12	99.23
	PC2	10.43	17.09	
	PC3	1.84	3.02	

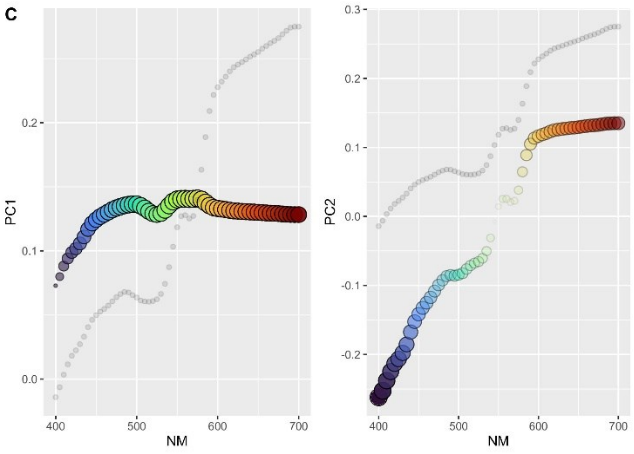
Supplementary Figure S1 Variation in factor loadings of the principal components with wavelength for blue/violet body stripe (A), yellow stripe in the tail fin (B) and yellow stripe in the anal fin (C). Only the principal components with eigenvalues >1 for all the stripes analysed are showed (see table S1). In all plots it's shown, in grey, the mean spectra of all males for the specific stripe, on which PCAs were performed. PCA of colour spectra allows to identify the wavelength regions of the spectrum that contribute the most to observed variation, by examining the PCs factor loadings (PC1 and 2 on the Y axis of the plots below) across the spectrum (here, from 400 to 700 NM, on the X axis).

PC1 accounts for the highest percentage of variation for all colour stripes (> 79%, see table S1) and is always positively and consistently loaded across all wavelengths, representing overall brightness.

PC2 is positively loaded by the 400-500 nm range and negatively loaded by the 500-700 nm range for the blue/violet stripe (A). Hence, PC2 provides an indication of the differences in the relative amount of short vs. long wavelength reflected from a blue stripe (blue chroma): high PC2 values indicate a strong violet-blue intensity and a lower green to red intensity. Conversely, PC2 is negatively loaded by the 400-550 nm range and positively (and less strongly) loaded by the 550-700 nm range for both the yellow stripes (B and C). Here, high PC2 values indicate a strong yellow-red intensity and a lower purple-blue intensity (yellow chroma).







Supplementary table S2 Results of the correlations between pre- and post-mating male traits. In black the correlation coefficient R and in red, below, the probability of correlation (p-value); in bold correlations with p-value >0.001.

	VAP	*VSL*	*VCL*	*LIN*	*MOT*	*LONG*	*M_SL*	*PC1B*	*PC2B*	*PC1YT*	*PC2YT*	*PC1YF*	*PC2YF*
VAP	-	**0.97** **<.0001**	**0.98** **<.0001**	0.07 0.514	**0.64** **<.0001**	**0.66** **<.0001**	-0.10 0.358	-0.03 0.824	-0.11 0.335	0.02 0.833	0.15 0.190	-0.04 0.694	0.25 0.024
VSL	**0.97** **<.0001**	-	**0.93** **<.0001**	0.29 0.009	**0.59** **<.0001**	**0.63** **<.0001**	-0.17 0.132	0.02 0.879	-0.14 0.201	0.02 0.852	0.09 0.446	0.05 0.668	0.29 0.010
VCL	**0.98** **<.0001**	**0.93** **<.0001**	-	-0.07 0.534	**0.65** **<.0001**	**0.70** **<.0001**	-0.03 0.765	-0.02 0.828	-0.08 0.460	0.05 0.689	0.14 0.215	-0.02 0.834	0.27 0.017
LIN	0.07 0.514	0.29 0.009	-0.07 0.534	-	-0.12 0.294	-0.10 0.387	**-0.37** **<.001**	0.09 0.427	-0.18 0.106	-0.09 0.430	-0.13 0.245	0.20 0.080	0.10 0.361
MOT	**0.64** **<.0001**	0.59 **<.0001**	0.65 **<.0001**	-0.12 0.294	-	**0.59** **<.0001**	-0.18 0.107	0.15 0.195	-0.07 0.511	0.11 0.342	-0.09 0.414	0.13 0.260	0.16 0.162
LONG	**0.66** **<.0001**	**0.63** **<.0001**	**0.70** **<.0001**	-0.10 0.387	**0.59** **<.0001**	-	-0.09 0.443	0.04 0.757	-0.03 0.769	-0.04 0.751	0.03 0.782	0.05 0.645	0.08 0.466
M_SL	-0.10 0.358	-0.17 0.132	-0.03 0.765	**-0.37** **<.001**	-0.18 0.107	-0.09 0.443	-	-0.27 0.014	0.09 0.411	-0.20 0.082	0.07 0.516	-0.05 0.691	-0.06 0.609
PC1B	-0.03 0.824	0.02 0.879	-0.02 0.828	0.09 0.427	0.15 0.195	0.04 0.757	-0.27 0.014	-	-0.06 0.574	**0.35** **0.001**	-0.18 0.120	**0.47** **<.0001**	-0.17 0.129
PC2B	-0.11 0.335	-0.14 0.201	-0.08 0.460	-0.18 0.106	-0.07 0.511	-0.03 0.769	0.09 0.411	-0.06 0.574	-	-0.08 0.502	**-0.40** **<.001**	-0.06 0.571	-0.16 0.151
PC1YT	0.02 0.833	0.02 0.852	0.05 0.689	-0.09 0.430	0.11 0.342	-0.04 0.751	-0.20 0.082	**0.35** **0.001**	-0.08 0.502	-	-0.02 0.864	0.31 0.005	0.11 0.334
PC2YT	0.15 0.190	0.09 0.446	0.14 0.215	-0.13 0.245	-0.09 0.414	0.03 0.782	0.07 0.516	-0.18 0.120	**-0.40** **<.001**	-0.02 0.864	-	-0.35 0.002	0.23 0.041
PC1YF	-0.04 0.694	0.05 0.668	-0.02 0.834	0.20 0.080	0.13 0.260	0.05 0.645	-0.05 0.691	**0.47** **<.0001**	-0.06 0.571	0.31 0.005	-0.35 0.002	-	0.01 0.960
PC2YF	0.25 0.024	0.29 0.010	0.27 0.017	0.10 0.361	0.16 0.162	0.08 0.466	-0.06 0.609	-0.17 0.129	-0.16 0.151	0.11 0.334	0.23 0.041	0.01 0.960	-

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4. Paper III

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

Female reproductive fluid concentrations drive sperm performance of alternative male phenotypes in an external fertilizer

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ABSTRACT

There is growing evidence that the female reproductive fluid (FRF) plays an important role in cryptic female choice through its differential effect on the performance of sperm from different males. In a natural spawning event, the male(s) may release ejaculate closer or further away from the spawning female. If the relative spatial proximity of competing males reflects the female pre-mating preference towards those males, then favoured males will encounter higher concentrations of FRF than unpreferred males. Despite this being a common situation in many external fertilizers, whether different concentrations of FRF can differentially influence the sperm performance of distinct male phenotypes (favoured and unfavoured by the female) remains to be elucidated. Here, we tested this hypothesis using the grass goby (*Zosterisessor ophiocephalus)*, a fish with distinct territorial-sneaker reproductive tactics and female pre-mating preference towards territorial males, that consequently mate in an advantaged position and whose sperm experience higher concentrations of FRF. Our findings revealed a differential concentration-dependent effect of FRF over sneaker and territorial sperm performance. Through their fluid, females seem to favour sperm depending on their proximity to the eggs during spawning. The ability to release sperm close to the eggs is a prerogative of territorial males, but FRF can likewise advantage sperm of those sneakers that are able to get closer, allowing flexibility in the direction of female post-mating choice.

INTRODUCTION

Decades of research on sexual selection have shown that polyandry, i.e. females mating with multiple males within the same reproductive episode, is a widespread phenomenon in many different species across the animal kingdom (Simmons, 2005). One of the most intriguing consequences of polyandry is the possibility for sexual selection to continue after mating, in the form of post-mating sexual selection (Birkhead & Pizzari, 2002). Post-mating episodes of selection include both intra-sexual sperm competition (i.e. the competition of sperm from two or more males to fertilize the same batch of eggs; Parker, 1970) and inter-sexual cryptic female choice (Thornhill, 1983; Eberhard, 1996). The latter occurs whenever polyandrous females can bias sperm utilization during and/or after mating. Unlike sperm competition, that has been extensively studied and recognized as a powerful force shaping male behaviour, morphology, and physiology (Birkhead & Møller, 1998; Simmons, 2001; Simmons & Wedell, 2020; Fitzpatrick, 2020; Birkhead & Montgomerie, 2020), cryptic female choice has remained for a long time the least investigated and understood mechanism of sexual selection (Firman *et al.,* 2017). This is mostly due to the experimental challenges associated to the study of these cryptic processes, and to the difficulties in disentangling their effects from those driven by sperm competition (Firman *et al.,* 2017; Thornhill, 1983). Although multiple routes of cryptic female choice, acting at different stages of the reproductive process (from right after mating to the moment of fertilization), have been identified (Eberhard, 1996), the mechanistic bases of these processes often remain obscure, particularly in externally fertilizing organisms (Kekalainen & Evans, 2018). However, since in externally fertilizing species females often have limited control over the male competitors participating in the reproductive event, sexual selection is expected to be particularly intense in its post-mating component, favouring the evolution of processes of cryptic female choice (Evans & Sherman, 2013). Recently, female reproductive fluid (hereafter FRF) has been suggested to be a possible key mediator of cryptic female choice in both internal and external fertilizers (Firman *et al.,* 2017). This fluid surrounds the eggs before and during fertilization and is kept inside the female reproductive tract in internal fertilizers or released along with the eggs in external fertilizers (Zadmajid *et al.,* 2019). Here, we adopt the term FRF (as in Gasparini *et al.,* 2020) to avoid specific taxa-related terminology since this fluid can have different origins (ovarian, oviductal, follicular and/or coelomic), but is in any case the medium with which sperm interact during fertilization (Zadmajid *et al.,* 2019; Gasparini *et al.,* 2020). FRF has been shown to positively affect various sperm performance traits, typically associated with sperm competition success, across a variety of different species, including a number of external fertilizers. In fish for example, FRF can mediate sperm attraction, enhance sperm viability, increase sperm velocity and sperm motility, prolong the duration of sperm motility, and modulate sperm trajectory and propulsion efficiency (Urbach *et al.,* 2005; Eloffson *et al.,* 2006;

Rosengrave *et al.,* 2009; Butts *et al.,* 2012; Gasparini & Evans, 2013; Alonzo *et al.,* 2016; Lenhert *et al.,* 2016; Lymbery *et al.,* 2017; Poli *et al.,* 2019; Myers *et al.,* 2020). Moreover, sperm performance in presence of FRF can also depend on specific male-female interactions. A female, through its FRF, can therefore differentially influence ejaculates of different males, thereby exerting cryptic female choice (Gasparini & Pilastro, 2011; Alonzo *et al.,* 2016; Lenhert *et al.,* 2016; Lenhert *et al.,* 2017; Rosengrave *et al.,* 2016; Lymbery *et al.,* 2017; Poli *et al.,* 2019; Myers *et al.,* 2020). In externally fertilizing fish, for instance, FRF has been shown to favour ejaculates of unrelated males during fertilization to avoid inbreeding (Lenhert *et al.,* 2017) and to bias the fertilization success toward the preferred male phenotypes in a fish species characterized by alternative male reproductive tactics (Alonzo *et al.,* 2016).

Yet, many factors of the external fertilization process's ecology have been neglected in this context, first and foremost the proximity of the male competitors to the spawning female. It is a widely held assumption that how close a male is to the female while she releases her eggs is an important predictor of its paternity success, with the probability of fertilization increasing with the degree of male proximity to the female (Hutchings *et al.,* 1999; Blanchfield *et al.,* 2003; Stoltz & Neff, 2006; Egeland *et al.,* 2015; Poli *et al,* 2021). Growing evidence indicates that paternity success in externally fertilizing species is indeed affected by the relative position of the competing males and the female, and that females, when possible, select a spawning site close to their preferred mate (Li *et al.,* 2015; Poli *et al,* 2021). However, so far, no one has considered the implications of this for FRF-mediated post-mating sexual selection, specifically, whether different concentrations of FRF (experienced by different males) might change or amplify the paternity outcomes discussed above. During fertilization, sperm encounter a gradient of increasing FRF concentrations in their journey toward the eggs and this is crucial to localize the eggs. Not all the sperm encounter the same gradient, and, in particular, sperm from different males may experience different FRF concentrations due to their relative proximity to the female during spawning. The occurrence of such different concentrations of FRF may provide a mechanistic way for females to favour the preferred male at post-mating level via the FRF effects on sperm, if those effects are concentration dependent.

Among external fertilizers, species showing male alternative reproductive tactics are excellent study systems to assess the possible role of FRF concentrations in mediating directional cryptic female choice, because of the pronounced pre-mating female preference for one of the two male phenotypes (usually the territorial/nesting male) that, as a consequence, gains closer vicinity to the female during spawning. The territorial phenotype is strongly preferred by females because it secures them with direct and/or indirect benefits, but females cannot prevent opportunistic males (sneaker/satellite) from participating in the reproductive events (Oliveira *et al.,* 2008). Furthermore, opportunistic males often produce more sperm than territorials, to boost their post-mating success. Therefore, females might exhibit mechanisms of reinforcing their pre-mating preferences in the postcopulatory competition (Alonzo *et al.,* 2016). Since the territorial male usually ejaculates closer to the egg-laying female than the sneaker does (Oliveira *et al.,* 2008), sperm of males adopting different tactics usually experience different concentrations of FRF, depending on the male proximity to the female. Females might, therefore, exert a choice on the ejaculates of males adopting different reproductive tactics through the effect of different concentrations of FRF on their sperm performances.

Here, we test this hypothesis using the grass goby *Zosterisessor ophiocephalus* (Pallas), an externally fertilizing fish in which territorial/sneaker (or bourgeois/ parasitic, according to Taborsky, 1997) male tactics have been widely investigated (Scaggiante *et al.,* 1999; Mazzoldi *et al.,* 2000; Locatello *et al.,* 2007; Locatello *et al.,* 2013). In this species, territorial males during the breeding season dig and defend a nest (a burrow under the seagrass rhizomes), court females (that show a strong pre-mating preference for this phenotype) and perform parental care to the eggs. Territorial males release sperm in the form of mucous trails on the nest ceiling, where the females release eggs one at the time. By contrast, sneaker males try to steal some fertilization by entering the nest and releasing ejaculates during the spawning event (Scaggiante *et al.,* 1999; Mazzoldi *et al.,* 2000). The level of sperm competition is intense in this species (Scaggiante *et al.,* 1999; Mazzoldi *et al.,* 2000; Locatello *et al.,* 2007; Locatello *et al.,* 2013), and sneaker males invest disproportionately more in sperm production than territorial ones. However, the two male phenotypes do not differ in sperm velocity and viability (measured both in water and in their own seminal fluid; Locatello *et al.,* 2007). Under natural spawning conditions, sperm of sneaker males experience a lower concentration of FRF than those of territorials, as they are usually released in an unfavourable spatial position, further from the spawning female (Fig.1). We expect that females should strengthen their pre-mating mate choice, favouring the sperm of territorial males at the post-mating level. To test this prediction, we assessed whether the different concentrations of FRF experienced during spawning elicit differences in the sperm performances of sneaker and territorial males. We measured a range of sperm motility traits in territorial and sneaker males in presence of different concentrations of FRF (0%, 10%, 20%) that mimicked those encountered by the two male phenotypes during the spawning event. We used the FRF of the same female with sperm from two males with different tactics, in order to disentangle tactic-specific effects of FRF from the ones driven by female's identity. Moreover, since in several species the sperm performance in presence of FRF has been shown to depend on specific male-female interactions, that might be influenced by female status/quality (Cotton *et al.,* 2006), we performed a second experiment (with a full factorial block design) using the higher FRF concentration to test whether FRF of different females could differentially affect the sperm motility of males of the two alternative tactics. In doing this we also checked if the FRF effect was eventually influenced by some female condition parameters and/or by the time of the reproductive season.

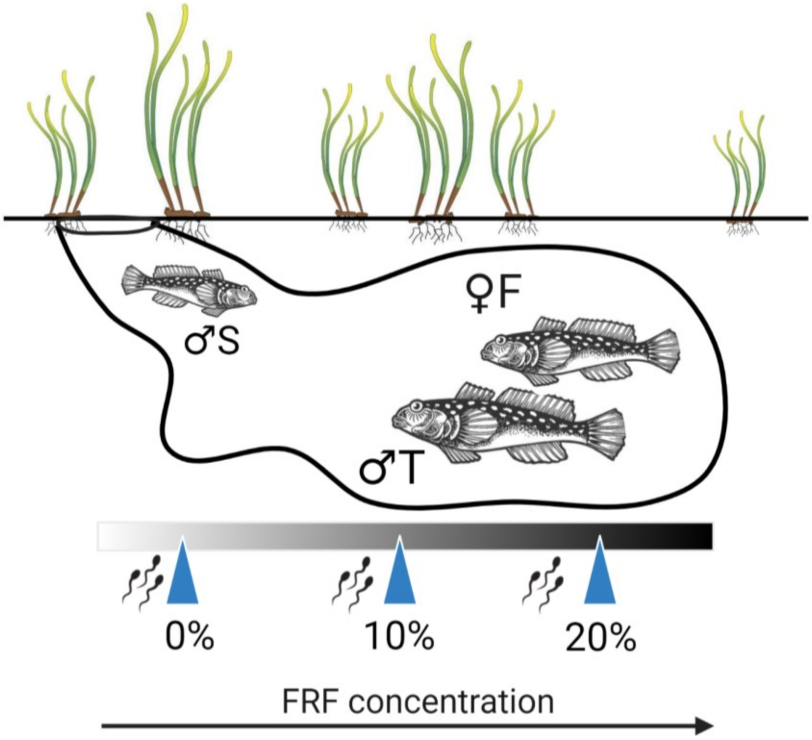

Figure 1. Schematic representation of a territorial male's nest, illustrating the (simplified) positions of the competing males and the female during spawning, together with the pattern of FRF concentration inside the nest. T: territorial male; S: sneaker male; F: female. Created with Biorender.com.

MATERIALS AND METHODS

Animal sampling and handling

Grass goby females, territorial and sneaker males were sampled, in nests full of eggs, in the Venetian Lagoon during their breeding season (March–May 2021) and transported to the Hydrobiological Station "Umberto D'Ancona" in Chioggia (Venice, Italy). In the field we performed the initial attribution of male tactics, taking advantage of previous information on the occurrence and size distribution of males performing either as territorial or sneaker (Mazzoldi *et al.,* 2000). In particular, to avoid determination mistakes, we categorized male tactics using the extreme values of size distribution, discarding individuals of intermediate size (11-18 cm) (Mazzoldi *et al.,* 2000). Thus, sneakers were considered as such only if a male larger than 18 cm was present in the nest where they were sampled and if their size was lower than 11 cm. Overall, the total length range of individuals used in our experiments was 6-11 cm for sneakers vs. 18-23 cm for territorials.

All individuals were kept for a maximum of 5 days in separate tanks with continuous water exchange (20°C ± 1°C) under a 14:10 artificial light-dark cycle and fed daily with fresh mussel. Prior to gametes' collection all individuals were anaesthetized in a water solution of MS 222 (tricaine sulphate; Sandoz), measured (SL = distance between the snout and the base of the tail fin) and weighed. Males' field categorization as territorial or sneaker was then validated according to the characteristics of their ejaculate trails (fluid and white in sneaker males, because of the high sperm content, and dense and opaque in territorial males, because of the lower sperm count and the higher mucin content; Scaggiante *et al.,* 1999) and to their sperm production (sperm number of sneakers = 1443975 ± 128167, territorials = 512030 ± 31662). After gametes' collection, all individuals were released, unharmed, at the site of collection.

Experimental design

Exp. A – Effect of different concentrations of FRF on male tactics

We tested the effect of the presence and concentration of FRF on sperm performance of territorial and sneaker males. Sperm of each male were exposed to three different treatments: 0% FRF (seawater only), 10% of FRF, and 20% of FRF in seawater. The real amount of FRF surrounding the eggs is difficult to estimate, as it continuously changes with time since egg deposition lasts for several hours, in nests containing a water volume of around 17.8 l (Mazzoldi *et al.,* 2000). However, a FRF 20% dilution is likely a plausible average concentration experienced by territorial male sperm, that are released from sperm trails laid in close proximity to eggs. 20% FRF dilution is also the concentration used in similar studies in other externally fertilizing fish species (Butts *et al.,* 2012; Lenhert *et al.,* 2016; Poli *et al.,* 2019). In contrast, sneaker males entering the nest are usually kept at a distance from the egg laying females by territorial males (Scaggiante *et al.,* 2005). Considering the nest volume and the amount of fluid released by the females we assume that, commonly, the sperm of sneakers might experience a range of FRF dilution from 0% to 10%. Thus, we measured sperm motility traits in 0%, 10%, and 20% concentrations of FRF in order to assess the sperm performance of the males in conditions mimicking those experienced by the sneaker and territorial males. We collected FRF from 15 ready-to-spawn females and ejaculates from 15 territorial and 15 sneaker males (see gametes and FRF collection below) and performed computer assisted sperm analysis (CASA) in the three different treatments (0%, 10%, and 20%) in randomised order for both male tactics.

Exp B. Effect of male reproductive tactic, female identity, and their interaction

We tested whether different females favour the performance of sperm of a specific tactic (interaction female identity x male reproductive tactic), while controlling for some female condition and environmental parameters (female size, total volume of FRF produced and time of the reproductive season). We applied a full-factorial block design in which the performance of sperm of 2 males of each tactic (2 territorials, 2 sneakers) were tested in presence of the FRF of 2 different females (see Figure 2). The sperm of each male, either territorial or sneaker, were tested with the FRF of two different females, and the FRF of the same female was used for 4 males, 2 sneakers and 2 territorials. The use of a different female for each pair of a territorial and a sneaker male, and of two different territorial or sneaker males (2 pairs) allowed to reveal the potential effect of the interaction between female identity and male tactic while controlling for individual interactions (female identity x male identity). We performed a total of 10 blocks (20 females, 20 sneaker males, 20 territorial males). In this experiment, FRF was diluted at 20% in filtered seawater, following previous studies on externally fertilizing fish species (Butts *et al.,* 2012; Lenhert *et al.,* 2016; Poli *et al.,* 2019).

The repeatability of sperm traits in FRF (10% and 20%) was checked in a separate trial using 10 males (5 sneaker and 5 territorials) and 10 females, with two replicates for each male and for each FRF concentration. The repeatability of sperm motility traits in water was already well documented in previous studies (Locatello *et al.,* 2007).

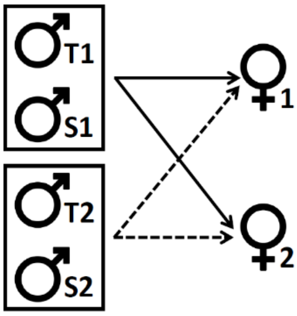

Figure 2. Schematic representation of one block from the full-factorial experiment (experiment B), composed of 2 females and 2 males for each mating tactic (4 males total).

Gametes and FRF collection

Eggs were obtained from anaesthetized ready-to-spawn females through a gentle pressure on their swollen abdomen (previously dried to prevent accidental activation of the eggs by water) and collected on standard microscope slides. The FRF surrounding the eggs was collected with a Gilson P2 micropipette (mean fluid volume per female: 23.6 µl ± 2.4) and preserved at -80°C until 1 hour before analysis, when it was thawed and diluted with filtered seawater at 20°C ± 1°C to the concentration of interest (10 or 20% according to the experiment). FRF was maintained in ice until analysis. To obtain the 10% and the 20% solution, 2 or 4 ul of FRF were diluted with 18 or 16 ul of solution (seawater + sperm), respectively.

Ejaculates were obtained through a gentle pressure on the abdomen (previously dried to avoid accidental activation of sperm by water) of anaesthetized males and collected with a Gilson pipette. Ejaculate samples were centrifuged at 13.300g for 3 min at 4°C to separate sperm from the supernatant seminal fluid (mean fluid volume: territorial: 93.6 µl + 9.8; sneaker: 16.4 µl + 1.8) and sperm cells were re-suspended in an extender inactivating medium (3.5 g l^{-1} NaCl, 0.11 g l^{-1} KCl, 0.39 g l^{-1} CaCl2, 1.23gl^{-1} MgCl2, 1.68gl^{-1} NaHCO3, 0.08gl^{-1} glucose, pH 7.7; Fauvel *et al.,* 1999). FRF and sperm samples were maintained on ice until analysis (within 1 h from collection). As sperm concentration varies among males and is significantly higher in sneakers than territorials, the volume of inactivating solution was individually adjusted to reach a standardized final concentration of 77629 ± 1086 sperm µl^{-1} in inactivated samples (final measured concentration, the approximate target concentration being 75000 sperm µl^{-1}, following 40). Sperm number in each sample was assessed with a LUNA™ Automated Cell Counter (Logos Biosystems).

Sperm analysis

For each assay, 6 µl of inactivated sample were activated by adding 1 µl of male's own seminal fluid and 13 µl of (i) 0% FRF, or (ii) 10 % FRF or (iii) 20% FRF, in random order.

Activated sperm samples were incubated for 2 min, a time sufficient to ensure the homogeneous activation of sperm while avoiding exhaustion, since in this species sperm remain active for more than 30 min (Scaggiante *et al.,* 1999). Three µl of sample were then placed in a separate well on a 12-well multitest slide (MP Biomedicals, Aurora, OH) previously coated with 1%polyvinyl alcohol (Sigma-Aldrich), to avoid sperm sticking to the glass slide (Wilsonl-Leedy & Ingermann, 2007) and covered with a coverslip. Sperm velocity was measured using a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA). The following parameters were considered: curvilinear velocity (VCL, µm s^{-1}), linearity of the path (LIN= straight line velocity, VSL / VCL), beat-cross frequency (BCF), and sperm motility (proportion of motile cells over the total). Mean speed measurements were based on 132.31+1.73 sperm tracks per sample.

Statistical analysis

All analyses were conducted using R v 3.6.3 (R core team, 2020). Repeatability was tested using the “rptR” package with Gaussian distribution for the variables VCL, LIN and BCF, and Proportion distribution for sperm motility, and based on 1000 permutations. All means are shown with associated standard error.

In both experiment VCL, LIN and BCF were analysed using linear mixed effect models (“lmer” function of the “lme4” package), while sperm motility was analysed with a generalized linear mixed effect model (“glmer” function of the “lme4” package) assuming a binomial error distribution and logit link function. In experiment A we tested the effect of the treatment (0% FRF, 10% FRF, 20% FRF), of male tactic, and of their interaction by including in the mixed effect models the treatment and the male mating tactic as fixed factors and the male identity and the female identity as random factors with fixed intercept. In experiment B the mixed effect models included male mating tactic as a fixed factor and male identity, female identity and the interaction between male mating tactic and female identity as random factors with fixed intercept. When a significant interaction male tactic * female identity was detected, female size, volume of FRF produced and time of the reproductive season were added in the model as covariates, to test for a potential influence of the female condition.

The associated P-values of the fixed factors were obtained from the “anova” function of the “lmerTest” package using Satterthwaite’s approximation to calculate the denominator degrees of freedom. *P* values for the random factors were obtained by likelihood ratio tests of the full model against the model with the specific random factor removed. Post-hoc pairwise comparisons were performed with the function lsmeans (package “lsmeans”), applying a Holm correction for multiple comparisons. Assumptions of the linear models were checked by inspection of residuals’ distribution, using the package “DHARMa”. Overdispersion in the generalized linear models was checked with the function “check_overdispersion” (library “performance”). We detected overdispersion for the first glm (experiment A) but not for the second (experiment B - dispersion ratio =0.864, Pearson's Chi-Squared =64.778, p-value =0.794). To account for overdispersion in the first glm we included an observation level random effect (test results after random effect inclusion: dispersion ratio = 0.195, Pearson's Chi-Squared = 14.656, p-value =1).

RESULTS

Repeatability

Repeatability of sperm performances in FRF was high at both FRF concentrations and for all sperm traits considered ($p<0.001$ for all traits at both concentrations, for details see table S1 of the supplementary materials).

Exp. A – Effect of different concentrations of FRF on male tactics

Sperm curvilinear velocity (VCL) was affected by treatment ($F_{2,3.9} = 8.21$, $p = 0.04$), but not by the male reproductive tactic ($F_{1,22.6} = 0.58$, $p = 0.45$), or by the interaction between treatment and tactic ($F_{2,47.2} = 0.77$, $p = 0.47$). In both tactics sperm velocity increased from 0% (seawater) to 10% and 20%, with the increase between the 10% and the 20% FRF treatments seemingly more pronounced in territorial males (figure 3a, table 1), but with no significant difference between tactics.

In presence of both concentrations of FRF, sperm linearity (LIN) decreased compared to 0% (seawater), with no difference between 10% and 20% FRF, for both male tactics (figure 3b, table 1), even though the effect of the treatment was found to be marginally non-significant ($F_{2,2} = 14.16$, $p = 0.07$). There was no effect of male reproductive tactic ($F_{1,24.6} = 0.09$, $p = 0.76$), or of the interaction between treatment and tactic ($F_{2,47} = 0.41$, $p = 0.67$).

Similarly, sperm beat cross frequency (BCF) decreased from 0% (seawater) to 10% and 20% FRF, with no difference between 10% and 20% FRF for both male tactics (figure 3c, table 1). Again, the effect of treatment was marginally not significant ($F_{2,2} = 13.88$, $p = 0.07$), the effect of the male reproductive tactic was not significant ($F_{1,24.42} = 0.66$, $p = 0.42$) and neither was the interaction between treatment and tactic ($F_{2,47.14} = 0.16$, $p = 0.85$).

Interestingly, sperm motility (MOT, i.e., the proportion of motile sperm) was significantly affected by the treatment ($\chi^2_2 = 39.43$, $p < 0.001$), and by the interaction between treatment and tactic ($\chi^2_2 = 7.09$, $p = 0.03$). There was no effect of the male reproductive tactic ($\chi^2_1 = 0.20$, $p = 0.66$). These results indicate how increasing concentrations of FRF mediated an improvement in sperm motility for both male tactics, but with a different trend in the two tactics (figure 3d, table 1). It is specifically in the comparison between 0% and 10% FRF that the effect significantly differs between tactics (interaction treatment*tactic: $\chi^2_1 = 6.78$, $p=0.009$). Post-hoc pairwise comparisons showed, indeed, that FRF significantly affect sperm motility of territorial males already at a low concentration (10%), whereas sperm motility of sneaker males was not affected by the lower concentration of FRF (10%) (table 1). Then, when the concentration of FRF reaches 20% (mimicking close proximity to the eggs), also sneaker sperm motility benefits of FRF similarly to territorial sperm. Sneaker sperm indeed showed significantly higher motility at 20% compared to 10% FRF (table 1).

Considering the most common spawning situation observed in nature (territorial male in close proximity to the female and sneaker further away) we also compared the sperm motility of sneaker males at 0 and 10% FRF concentration with the sperm motility of territorial males at 20% FRF concentration. The results indicate a significant difference in the sperm motility of males of the alternative tactics when they are at varying distances from the female, more pronounced as the gap in distance between the males increases (sneaker 0 vs territorial 20: z= -2.36, p=0.037; sneaker 10 vs territorial 20: z= -1.93, p=0.053).

For none of the sperm traits analysed we evidenced significant differences between the two tactics within each treatment (all Pr(>|t|) > 0.1, for details see table S2 of the supplementary materials).

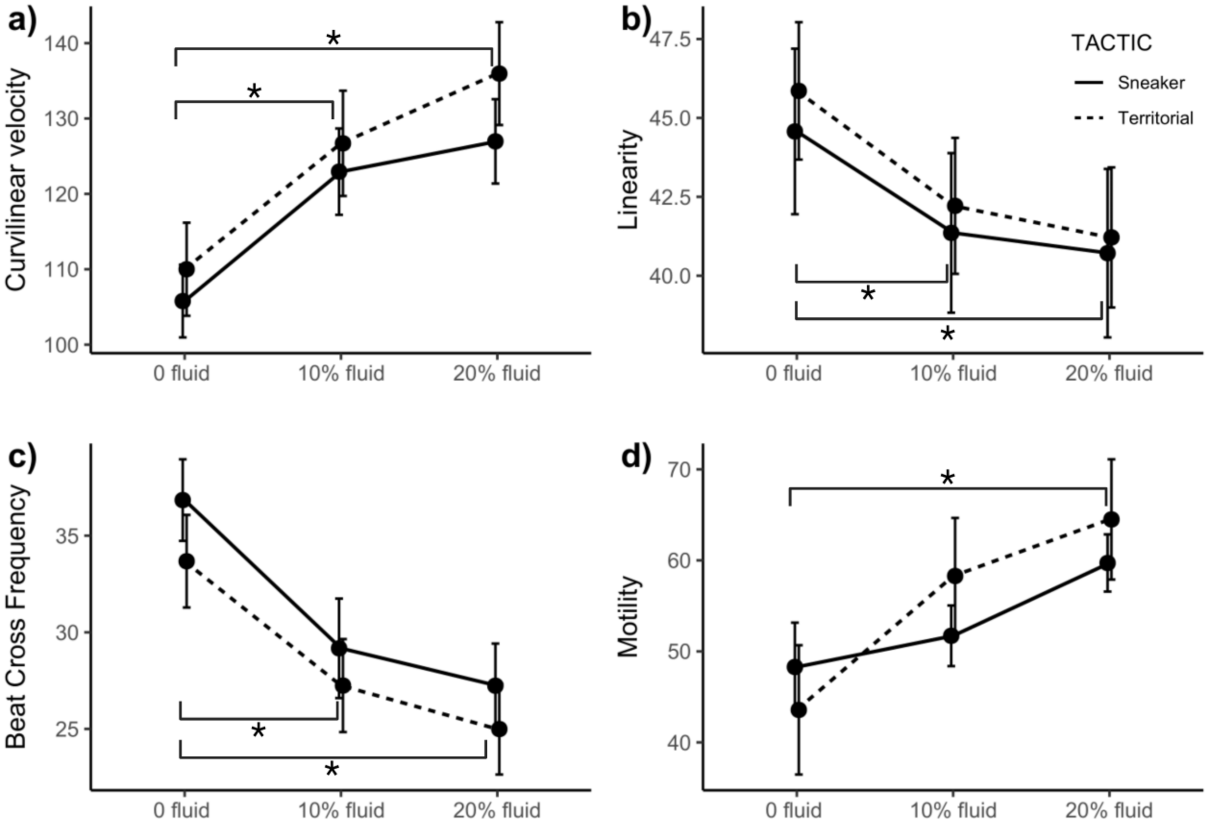

Figure 3. Effect of different concentrations of FRF compared with seawater (0 fluid) on different sperm traits of sneaker (solid line) and territorial males (dashed line) (means ± SE): a) sperm curvilinear velocity VCL (μm/s), b) sperm linearity LIN, c) sperm beat-cross frequency BCF, d) percentage of motile sperm (sperm motility MOT). Asterisks indicate significant differences among treatments levels for both male tactics, as evidenced by pairwise comparisons (table 1).

Table 1. Pairwise comparisons of FRF effects on all the sperm traits across all treatment levels' combinations, for each male reproductive tactic (df = 26).

		SNEAKER		TERRITORIAL	
Sperm Trait	contrast	t/z-ratio	P	t/z-ratio	P
Curvilinear Velocity	0-10% FRF	-4.99	**<0.001**	-4.761	**<0.001**
	0-20% FRF	-6.16	**<0.001**	-7.398	**<0.001**
	10-20% FRF	-1.17	0.253	-2.637	**0.014**
Linearity	0-10% FRF	2.41	**0.047**	4.45	**<0.001**
	0-20% FRF	2.89	**0.023**	5.67	**<0.001**
	10-20% FRF	0.48	0.634	1.22	0.233
Beat Cross Frequency	0-10% FRF	4.54	**<0.001**	4.13	**<0.001**
	0-20% FRF	5.69	**<0.001**	5.58	**<0.001**
	10-20% FRF	1.15	0.26	1.44	0.16
Motility	0-10% FRF	-1.08	0.28	-3.53	**<0.001**
	0-20% FRF	-3.67	**<0.001**	-5.1	**<0.001**
	10-20% FRF	-2.61	**0.018**	-1.59	0.113

Exp B. Effect of male reproductive tactic, female identity, and their interaction

From the full-factorial experiment (Figure 2), we found no effect of male reproductive tactic for any of the sperm traits considered (table 2), as expected from the previous experiment. Sperm velocity, linearity and beat cross frequency were not affected neither by female identity, nor by the interaction between female identity and male reproductive tactic (table 2). Sperm motility, however, was significantly affected by female identity, and we found a significant effect also of the interaction between female identity and male reproductive tactic (table 2). This suggest that the fluid of different females differentially influenced the proportion of motile sperm of a specific male reproductive tactic. We therefore assessed whether the female condition (body size and volume of FRF produced) or the time of the reproductive season (date of the experiment) were driving the observed effects on sperm motility mediated by the female identity, but none of these covariates was found to be significant (all Pr(>|z|) > 0.3).

Finally, the male identity had a significant effect on all these traits, except for sperm beat-cross frequency (table 2), meaning that different males showed intrinsically different sperm performances in terms of velocity, trajectory, and motility.

Table 2. Estimates and significance levels for the fixed factor "male tactic" and the random factors of linear mixed effect models: Female ID, interaction between Female ID and Male tactic and Male ID for all the sperm traits considered.

	Male tactic		Female ID		Female ID x male tactic		Male_ID	
Sperm trait	t/z value	P	χ2	P	χ2	P	χ2	P
Curvilinear Velocity	0.93	0.359	0.31	0.855	0	0.999	24.73	**<0.001**
Linearity	1.94	0.061	0.78	0.547	0	0.999	23.00	**<0.001**
Beat Cross Frequency	1.10	0.287	0.15	0.926	0	0.999	1.28	0.258
Motility	-0.44	0.657	25.29	**<0.001**	3.95	**0.046**	188.12	**<0.001**

DISCUSSION

Our results showed that FRF has positive effects on sperm performance, corroborating a growing number of studies on the topic (see Zadmajid *et al.,* 2019; Gasparini *et al.,* 2020). However, only few studies have investigated whether the FRF has a differential effect on alternative reproductive tactics (Alonzo *et al.,* 2016; Lenhert *et al.,* 2017), and none have explored the possible role of FRF concentration on such effect. In our study, FRF presence has similar effects on the sperm performances of both male phenotypes, positive on sperm velocity and proportion of motile sperm, negative on sperm linearity and beat cross frequency. However, when taking into consideration different concentrations of FRF, an interaction between the treatment (FRF concentration) and the male tactic emerged for sperm motility (percentage of motile sperm). In particular, the presence of FRF at lower concentrations improves the sperm motility of territorial males but not of sneaker ones. However, the latter is enhanced by higher FRF concentrations, reaching that of territorial male sperm. This suggests that a female effect on sperm of different male phenotypes can be mediated by the different concentrations of the FRF experienced by the competing ejaculates in the race for fertilization. Thus, females might exert a cryptic choice, via their FRF effects on sperm traits, based on the proximity of males when releasing their ejaculates rather than on the male tactics *per se*.

In nature, sneakers of the grass goby usually release their sperm at the nest entrance, thus their sperm encounter low concentrations of FRF, or no FRF at all (Fig. 1). In getting closer to the female (simulated by a 10% FRF concentration) sneakers can only partially benefit of FRF with an increase in sperm velocity but not in sperm motility. On the other hand, territorial males experience higher FRF concentrations, as they lay sperm trails very close to the eggs (in our design simulated with 20% FRF). Territorial males benefit from increased proximity to females in terms of both sperm velocity and motility, with a greater disparity in sperm motility between the alternative tactics when the difference in their distance from the female is greater. In such a common scenario the direction of female post-mating choice seems, therefore, congruent with the pre-mating choice: the differential effect of FRF might influence the outcome of sperm competition by decreasing the numerical advantage of the sneakers, who release more sperm but further away from the female compared to territorial males (Mazzoldi *et al.,* 2000). Nonetheless, should a sneaker male be able to get close enough to the female to benefit from the same FRF concentration as the territorial male, then both tactics will likely have the same chances at fertilization, or the fertilization game might be even skewed towards sneakers, since, in addition, they release a higher number of sperm (Mazzoldi *et al.,* 2000). Furthermore, it is already known that sneaker males have the ability to exploit the seminal fluid of competitor territorial males, whose sperm performance is in turn penalized by the presence of sneaker seminal fluid (Locatello *et al.,* 2013). Such evolutionary dynamics of ejaculate exploitation and impairment in sperm competition mediated by male seminal fluid, together with the differential effect of FRF here described, makes the grass goby an ideal study system to investigate the combined selective pressures of both sperm competition and cryptic female choice. The concentration-based effect of FRF is of particular interest also because the two distinct territorial-sneaker mating tactics in the grass goby are expressed as an ontogenetic gradient (Scaggiante *et al.,* 2004). In this species, younger males can indeed switch from sneaking tactic to territorial one in favourable social conditions, such as nest and ripe females' availability, with the opportunity to spawn and perform parental care (Scaggiante *et al.,* 2004).

Females might therefore gain indirect genetic benefits not only by having their eggs fertilized by the territorial male, but also by a successful sneaker male (the one able to get as close as the territorial to the spawning female). Whether the position a sneaker male is able to obtain during fertilization is associated with its quality, and hence indirect genetic benefits for the female, would require specific follow up experiments.

In natural conditions, therefore, the ideal post-mating strategy for females may be to use the proxy of male spatial positioning instead of discriminating among alternative mating tactics *per se*. Grass goby females, overall, appear to be flexible in their pre- and post-mating choices. At the pre-mating level they strongly favour territorial males, most likely because of the direct benefits associated with such preference, but at the post-mating level the direction of the choice is not that fixed. If sneaker males are successful enough to benefit from a high concentration of FRF they are provided with the same chances of fertilization as the preferred territorial male. This possibly suggests a shift in the relative importance of direct and indirect (genetic) benefits from the pre- to the post- mating events, with the former driving females in their pre-mating choice, and genetic benefits gaining relative more importance at the post-mating level, when the mate quality transcends the mating tactics adopted by males.

Our findings also highlight a significant effect of female identity and an interaction between female identity and male reproductive tactics on sperm motility. This indicates that different females affected differentially, via their FRF, the sperm motility of males of the two alternative reproductive tactics. This suggests that, in the grass goby, in which sperm of territorial and sneaker males have similar quality (Locatello *et al.,* 2007), the FRF did not consistently favour the sperm of a specific male phenotype. Our observations concur with the evidence of a widespread variability in female preferences across the animal kingdom (Cotton *et al.,* 2006; Rodrìguez, 2020; Rosenthal & Ryan, 2022). Preferences can be opposite in different females of the same population, and even in the same female over the course of life, suggesting adaptive flexibility in preferences (Rosenthal & Ryan, 2022), with females often getting more permissive as mates become scarce, risks increase, or time is short (Pennebaker *et al.,* 1979; Hedrick & Dill, 1993; Rand *et al.,* 1997).

Variation in mating decisions might often result from condition-dependent differences in female quality, and/or from context-dependent environmental factors, since mate choice is a costly behaviour in many species (Cotton *et al.,* 2006; Hingle *et al.,* 2001; Griggio & Hoi, 2010). Here, we controlled for some female parameters (female size, total volume of FRF produced and the date of the experiment) to understand whether the female condition or the time of the reproductive season were somehow driving the observed effects mediated by the female identity, but none of these covariates was found to be significant. Future studies are needed to shed light on the role of female physiological status, such as stress level and hormonal condition, in the influence exerted by the FRF on female post-mating preference.

Emerging evidence suggests that females may evaluate different aspects of mate quality in separate moments of the mating event: male phenotype and overall quality before mating, sperm quality and competitiveness afterwards (Rosengrave *et al.,* 2008; Evans & Garcia-Gonzalez, 2016; Firman *et al.,* 2017). The mechanism mediated by FRF described here provides a powerful example of this shift in male quality assessment from pre- to post-mating arenas. Moreover, we provide here the first evidence of a concentration-dependent effect of FRF that can be directional under specific circumstances (i.e., favouring only territorial males when they are the sole proximate males to the female), but also more permissive according to the quality of the partners involved (i.e., favouring also those sneakers that are able to get closer and benefit of higher FRF concentration).

Overall, this study adds a new piece to the puzzle of how FRF can mediate post-mating sexual selection in external fertilizers, unravelling the importance of experimentally simulating, as much as possible, the natural spawning conditions to better decipher the multiple factors that play in the complexity of the real world.

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Ethics

The experiments conducted herein comply with the current Italian laws (permission OPBA University of Padova no 39/2021).

Author contributions

All authors conceived the study, P.L. and R.M.B. designed the experiments, P.L. and L.L. developed the methods. P.L. collected and analysed the data and wrote the original draft. All authors reviewed and edited the draft and contributed to the final version of the manuscript.

Data accessibility

The datasets supporting this article are available on the figshare repository: https://doi.org/10.6084/m9.figshare.19984217.v1

SUPPLEMENTARY MATERIALS

Table S1. Results of repeatability analysis for the sperm traits of interest at the different concentrations of FRF.

		R	C.I.	p
Curvilinear velocity (VCL)	FRF 10%	0.999	0.995, 1	<0.001
	FRF 20%	0.998	0.993, 0.999	<0.001
Beat Cross Frequency (BCF)	FRF 10%	0.998	0.994, 0.999	<0.001
	FRF 20%	0.995	0.982, 0.999	<0.001
Linearity (LIN)	FRF 10%	0.995	0.977, 0.998	<0.001
	FRF 20%	0.993	0.977, 0.998	<0.001
Motility (MOT)	FRF 10%	0.073	0.018, 0.119	<0.001
	FRF 20%	0.037	0.013, 0.074	<0.001

Table S2. Effect of male mating tactic on the sperm traits of interest: results of lm (VCL,LIN,BCF ~ male tactic in water), lmer (VCL,LIN,BCF ~ male tactic with female identity as random factor in 10% and 20% FRF) and glmer (MOT ~ male tactic with an observation level random factor to account for overdispersion in water, + female identity as additional random factor in 10 and 20% FRF) for each treatment level.

Sperm trait	Treat. level	Estimate	Std. error	t/z value	Pr(>|t/z|)
VCL	water	4.22	7.83	0.54	0.59
	10% FRF	3.76	6.74	0.56	0.59
	20% FRF	8.99	6.41	1.40	0.18
LIN	water	1.28	3.41	0.38	0.71
	10% FRF	0.86	1.72	0.50	0.63
	20% FRF	0.50	1.55	0.32	0.75
BCF	water	-3.16	3.19	-0.99	0.33
	10% FRF	-1.93	2.95	-0.65	0.52
	20% FRF	-2.24	2.87	-0.78	0.45
MOT	water	-0.23	0.38	-0.6	0.55
	10% FRF	0.34	0.29	1.17	0.24
	20% FRF	0.31	0.33	0.93	0.35

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5. Paper IV

In preparation for submission to a peer-reviewed journal.

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

Female reproductive fluid and male seminal fluid: the non-gametic conflict for post-mating control

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ABSTRACT

Growing evidence shows that non-gametic components released by both males and females can play a major role in influencing sperm competition outcomes. Seminal fluid was shown to influence paternity success by affecting rival males' sperm performance, and in some species with male alternative reproductive tactics was reported to selectively decrease the fertilization success of males of the opposite tactic. Female reproductive fluid (FRF) has been proven to differentially influence ejaculates of different males and bias fertilization towards specific partners. Whether, and with what outcome, these two processes can intersect to influence sperm competition is still unknown. Here we explore this scenario in the grass goby (*Zosterisessor ophiocephalus),* a fish with territorial-sneaker reproductive tactics, where sneaker males can exploit the territorials' seminal fluid while penalizing territorial sperm performance with their own fluid. To test whether FRF can rebalance the ejaculate competition in favour of territorial males, we used in vitro fertilization with a seminal fluid mixture (territorial + sneaker), using increasing concentrations of FRF, to simulate the natural conditions that ejaculates encounter towards the eggs. Our findings revealed a differential effect of FRF on the fertilization success of the different tactics, in presence of a mixture of their seminal fluids. Such a process favours territorial ejaculates, possibly through the attenuation of the detrimental effects of sneaker seminal fluid, thus enabling females to regain control over the fertilization process.

INTRODUCTION

Polyandry (i.e., females mating with multiple males during the same reproductive event) is a taxonomically widespread phenomenon with important evolutionary implications. (Birkhead & Moller, 1998; Birkhead, 2000; Simmons, 2001; Griffith *et al.,* 2002). When females mate polyandrously, sexual selection can indeed continue after the mating event, up to the point of fertilization (Parker, 1984; Tregenza & Wedell, 2000). With the rise of post-mating sexual selection research, theoretical and empirical studies have historically focused on the competitive interplay between the sperm of different males, neglecting both the non-gametic components released with the gametes and the interaction between the sexes. Sperm competition, occurring whenever the ejaculates of rival males compete to fertilize the same eggs (Parker, 1970), quickly established itself as a pervasive evolutionary force able to shape male behaviour, physiology, and morphology, and to influence ejaculate production and allocation strategies (Birkhead & Moller, 1998; Simmons, 2001; Birkhead & Pizzari, 2002; Lüpold *et al.,* 2020; Parker, 2020). It was only later that seminal fluid started to attract attention in the context of sperm competition, primarily for its indirect influence over paternity success through its effects on female receptivity, oviposition rate and remating possibilities (Poiani, 2006; Chapman, 2008). More recently, seminal fluid was also shown to directly impact the outcome of sperm competition, by affecting the sperm performance of rival males. The effects exerted by seminal fluid on rival ejaculates can be positive, enhancing both one's own sperm competitiveness and that of rivals, but can also be negative, specifically decreasing the fertilization success of the competitors (Perry *et al.,* 2013). Male parasitism of rival ejaculates has been firstly found in some insects, with seminal fluid either equally improving own and rival sperm viability, (Hodgson & Hosken, 2006; Holman, 2009; Simmons & Beveridge, 2011) or, by contrast, incapacitating the sperm of rival males (Fry & Wilkinson, 2004; den Boer *et al.*, 2010). Interestingly, in two fish species with male alternative reproductive tactics (hereafter ARTs) a tactic-specific detrimental effect of seminal fluid on rival sperm performances was observed (Locatello *et al.,* 2013; Lewis & Pitcher, 2017). In the grass goby (*Zosterisessor ophiocephalus*) (Pallas), a species with a guard-sneaker mating system, sneaker males were shown to exploit the seminal fluid of competitor territorial males, whose sperm performance was in turn penalized by the presence of sneaker seminal fluid, both in terms of velocity and fertilization success (Locatello *et al.,* 2013). Similarly, a study on the Chinook salmon (*Oncorhynchus tshawytscha*) revealed that jack (parasitic tactic) seminal fluid could decrease hooknose (dominant tactic) sperm velocity, while the seminal fluid of hooknoses had no effect on the sperm performance of males of the opposite tactic (Lewis & Pitcher, 2017).

Species with ARTs represent the ideal model to investigate dynamics of sperm competition mediated by both gametic and non-gametic components, as males of the alternative tactics usually experience different levels of sperm competition and can allocate strategically both sperm and seminal fluid production in response (Oliveira *et al.,* 2008). Notably, sneaker/parasitic male seminal fluid detrimentally affecting sperm competitiveness of guard/dominant males goes against female pre-mating preferences. This sets the stage for a potential sexual conflict that could trigger co-evolutionary processes in which female post-mating mechanisms are expected to re-balance the competition in favour of initially preferred males.

Sperm competition processes always take place in a reproductive environment largely shaped by the females, even in external fertilizers, where fertilization, despite happening outside the female reproductive tract, is nonetheless influenced by the female reproductive fluid released along with the eggs. Female reproductive fluid (hereafter FRF) is the medium, surrounding the eggs before and during fertilization, with which sperm must come in contact with during fertilization (Gasparini *et al.,* 2020). FRF has been shown to differentially influence sperm competitiveness of different males, thereby mediating cryptic female choice, across a variety of different species (Urbach *et al*., 2005; Rosengrave *et al*., 2008; Evans *et al.,* 2013; Poli *et al*., 2019; Fitzpatrick *et al*., 2020). In fish, for instance, FRF has been shown to favour, during fertilization, ejaculates of unrelated (Gasparini & Pilastro, 2011), or more genetically compatible males (Lehnert *et al.,* 2017), and to bias fertilization toward the preferred male phenotype in a fish species with ARTs (Alonzo *et al.,* 2016).

Despite the increasing interest for the role of FRF in post-mating sexual selection, no one so far has ever explored the implications of FRF interacting with male seminal fluid during fertilization, and whether this might influence the battle for post-mating control.

Here, we use the grass goby *Z. ophiocephalus* to explore the potential of FRF to interfere with the dynamic of sperm competition impairment mediated by seminal fluid. This species exhibits external fertilization and male ARTs, with territorial males building and defending a nest during the breeding season, courting females, and providing parental care to the eggs, and sneaker males parasitizing the territorial spawnings by quickly releasing their ejaculates inside the nest to steal some fertilizations (Scaggiante *et al.,* 1999; Mazzoldi *et al.,* 2000). Females of the grass goby display a strong pre-mating preference for territorial males, that usually release sperm in the form of mucous trails on the nest ceiling, where females lay their eggs one at a time. Conversely, sneaker males usually release their fluid ejaculates from an unfavourable position, further away from the spawning female. To compensate for this positional disadvantage, and for the intense level of sperm competition, sneaker males invest disproportionately more in sperm production than territorials, that in turn invest more in seminal fluid production (Locatello *et al.,* 2007). As a result, territorial males produce ejaculates that slowly dilute in seawater, ensuring a constant supply of active sperm throughout the egg deposition process, so they can focus on nest defence and deter sneaking attempts (Scaggiante *et al.,* 2005).

A recent study revealed a differential concentration-dependent effect of FRF over both sneaker and territorial sperm performance, with sperm released in close proximity to female, thus encountering a higher FRF concentration, significantly increasing their velocity and motility (Pinzoni *et al.,* submitted). The ability to release sperm close to the eggs is a prerogative of territorial males. Thus, FRF could potentially serve as a mechanism to favour the territorial male phenotype at the post-mating level. However, as recently shown in this species, sneaker males reaching the proximity of territorial males gain a higher fertilization success by enjoying an increase in sperm performance exploiting the territorial fluid, and decreasing, with their seminal fluid, the territorials' sperm competitiveness (Locatello *et al.,* 2013).

The grass goby is therefore perfectly suited to tackle the question of FRF interference with male seminal fluid, with the goal of understanding whether FRF could re-balance the sperm competition to the female advantage and attenuate the detrimental effect of sneaker seminal fluid on territorials' sperm performance.

To do so, we designed an in vitro fertilization experiment where the ejaculates competition was simulated by mixing sneaker and territorial seminal fluid, and the sperm of males of the alternative tactics were subject to two different concentrations of FRF, to simulate the condition of natural competition that ejaculates inevitably encounter in their journey towards the eggs.

MATERIALS & METHODS

Grass goby females, territorial and sneaker males were sampled in the Venetian Lagoon during their breeding season (March–May 2022). In the field males were initially categorized as territorials or sneakers following Pinzoni *et al.,* (submitted). All individuals were then transferred to the Hydrobiological Station "Umberto D'Ancona" in Chioggia (Venice, Italy), where they were kept, for a maximum of 5 days, in separate tanks with continuous water exchange (20°C ± 1°C) under a 14:10 artificial light-dark cycle and fed daily with fresh mussel.

The day of the experiment, all individuals were anaesthetized in a water solution of MS 222 (tricaine sulphate; Sandoz), weighed and measured. Gametes were then collected following Locatello *et al.,* (2013). Briefly, eggs were obtained through a gentle pressure on the swollen abdomen of ready-to-spawn females and collected, surrounded by FRF, on acetate sheets of standard length. Ejaculates were obtained in the same way by gently pressing on the abdomen of males and collected with a Gilson pipette. Ejaculate samples were centrifuged at 13.300g for 3 min at 4°C to separate sperm from the supernatant seminal fluid and sperm cells were re-suspended in an extender inactivating medium (Fauvel *et al.,* 1999). The field attribution of males' mating tactics was then validated according to their ejaculates' characteristics (fluid and white in sneaker males, because of the high sperm content, and dense and opaque in territorial males, due to the lower sperm count and the higher mucin content) and sperm production (sperm number of sneakers = 1563647 ± 107969, territorials = 534012 ± 61890). Sperm number in each sample was assessed with a LUNA™ Automated Cell Counter (Logos Biosystems).

For each male, subsamples of 10 µl of the sperm solution were activated with 20 µl of marine filtered seawater and incubated for 2 min with 2 µl of seminal fluid (1 µl own seminal fluid + 1 µl of the rival of the opposite tactic). Sneakers' seminal fluid was pre-diluted 10-fold in filtered seawater before use to match the natural seminal fluid concentration of sneaker and territorial males (Locatello *et al.,* 2013). A volume of sperm solution containing 8 x 10^5 sperm cells was then taken, diluted to 50 µl with filtered seawater, and used for IVFs. These were performed by placing two acetate sheets, carrying the eggs of two different females, on the bottom of a glass beaker containing 500 or 250 ml of filtered seawater, simulating the two different scenarios of sperm being respectively distant or close from the female, as illustrated in the experimental design in Fig.1. Eggs were pooled from two different females to minimize the potential male-by-female interaction effects (Fitzpatrick *et al.,* 2012). A new group of 2 females was used for each sneaker-territorial pair, while the same 2 females randomly gave the pools of eggs for the different treatments performed on each pair (close/distant with respect to the female, for both males). Sperm were homogeneously deposited on the water surface with a Gilson pipette and let to fertilize the eggs for 15 min. Afterwards, the sheets were extracted, gently washed and placed in a new glass beaker with clean filtered seawater and oxygen supply. Fertilization success was checked at 4h post fertilization, when the complete lifting of chorion and the first stages of cellular division are clearly visible. For each IVF, 259.04 ± 2.25 eggs were used. Afterwards, all individuals were released at the site of collection unharmed.

Statistical analyses were conducted using R v 3.6.3 (44). The proportion of fertilized eggs across treatments was analysed using a linear mixed effect model ("lmer" function of the "lme4" package; Bates *et al.,* 2015). We tested the effect of the treatment ("high" and "low" concentrations of FRF), of male tactic, and of their interaction by including in the mixed effect models the treatment and the male mating tactic as fixed factors and the male and female identity as random factors with fixed intercept. The associated P-values of the fixed factors were obtained from the "anova" function of the "lmerTest" package (Kunzetsova *et al.,* 2017) using Satterthwaite's approximation to calculate the denominator degrees of freedom. Post-hoc pairwise comparisons were performed with the function lsmeans (package "lsmeans"; Lenth, 2016), applying a Tukey correction for multiple comparisons. The inspection of residuals' distribution using the package "DHARMa" indicated that the linear model met the assumptions (Hartig, 2020). All means are shown with associated standard error.

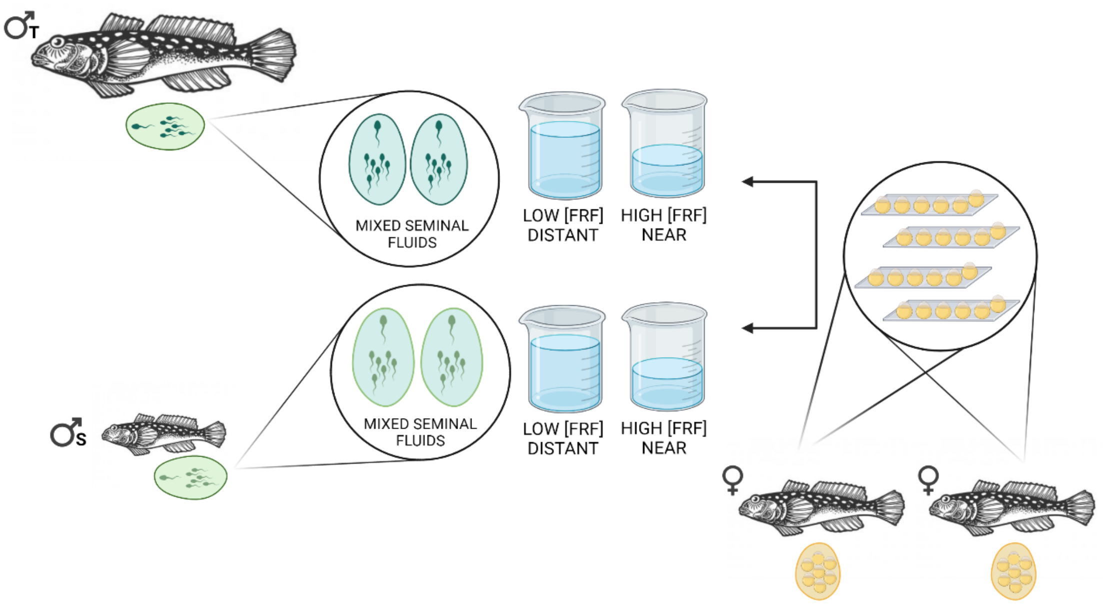

Figure 1. Schematic representation of experimental design - To test for the role of FRF in determining the sperm competition outcome of ARTs, we compared the fertilization success of sneaker and territorial males in presence of a mixture of both seminal fluids across two treatments: high and low concentration of FRF (simulating, respectively, being near the eggs, or distant from the eggs). For each replicate (consisting of one sneaker, one territorial, and two females) we collected the eggs (standardized number) of each female on 4 acetate sheets (total 8 sheets) that were randomly assigned to the different treatments. Then, using two glass beakers containing filtered marine seawater (with one volume twice as large as the other), we performed individual IVFs as described above. Fertilization success of each male across treatments was then individually recorded. Total number of replicates = 17. Made with Biorender.

RESULTS

We found a positive effect of treatment (increasing concentrations of FRF) ($F_{15.798}$ = 17.97, p<0.001), of male mating tactic ($F_{31.531}$ = 31.53, p = 0.002) and, notably, of their interaction ($F_{16.254}$ = 48.33, p<0.001) on fertilization success (in presence of a mixture of own seminal fluid and of seminal fluid of the opposite tactic). This indicates that males' fertilization success was differentially affected by the increasing of FRF concentration according to the mating tactic adopted (territorial or sneaker). Specifically, at lower FRF concentration (i.e., simulating a higher distance from the eggs) sneaker males were significantly more successful in fertilization that territorials, but this difference between tactics disappeared in presence of higher concentrations of FRF (i.e., simulating higher proximity to the eggs) thanks to the significant increase of territorial males' fertilization success (table 1, figure 2). Indeed, the increasing concentration of FRF significantly boosted the fertilization success of territorial males from 44.55 ± 2.74% to 56.84 ± 3.47%, whereas sneakers' fertilization success slightly decreased, although non significantly, from 66.17 ± 3.02% to 63.49 ± 2.99% (table 1, figure 2).

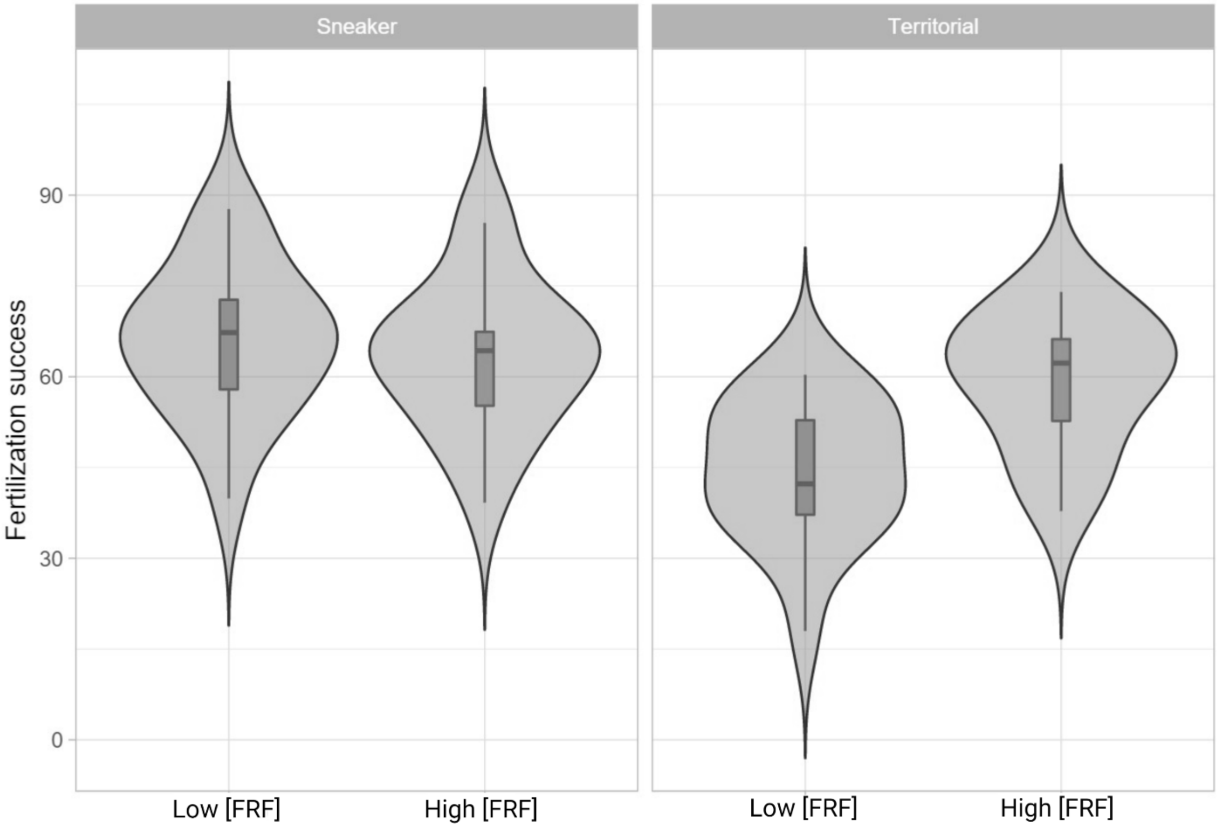

Figure 2. Effect of increasing concentrations of FRF on the fertilization success of territorial and sneaker males. Violin plots show the distribution of the variable "fertilization success" while box plots show the median, interquartile range, and upper and lower extremes of the variable. N = 17 territorials and 17 sneakers.

Table 1. Post-hoc pairwise comparisons of the effect of FRF increasing concentrations ([FRF]) on the fertilization success of sneaker and territorial males across all treatment*tactic combinations.

contrasts	*estimate*	*SE*	*t*	*p*	*95% CI*
Sneaker low [FRF]– Sneaker high [FRF]	0.027	0.016	1.717	0.332	-0.016 to 0.069
Sneaker low [FRF]– Territorial low [FRF]	0.216	0.043	5.006	**<.001**	**0.100 to 0.333**
Sneaker low [FRF]– Territorial high [FRF]	0.093	0.043	2.151	0.157	-0.024 to 0.210
Sneaker high [FRF]– Territorial low [FRF]	0.189	0.043	4.369	**<.001**	**0.073 to 0.306**
Sneaker high [FRF]– Territorial high [FRF]	0.066	0.043	1.536	0.428	-0.050 to 0.183
Territorial low [FRF]– Territorial high [FRF]	-0.123	0.016	-7.862	**<.001**	**-0.165 to -0.081**

DISCUSSION

Our results evidence a differential effect of increasing concentrations of FRF on the fertilization success of males adopting ARTs, in presence of seminal fluids of both competitors of the alternative tactics. In our experiment, in which we simulated the hypothetical concentration of FRF encountered by sperm when released at a higher distance or closer to the eggs, it emerges how at lower concentrations of FRF the fertilization success of sneaker males is significantly higher compared to territorials. This is in line with the results of the abovementioned study in which, by performing IVFs in the same volume of seawater (500 mL), therefore keeping constant the concentration of FRF, a tactic-specific effect of male seminal fluid on own and rival sperm performance was found, ultimately leading to a higher sneaker fertilization success in a scenario of mixed seminal fluids (Locatello *et al.,* 2013). Here, however, when the sperm get closer to the eggs and encounter increasing concentrations of FRF (a scenario that we simulated using half the volume of seawater) territorial males get a boost in their fertilization success, allowing them to “catch up” on sneaker males, that, at the opposite, do not experience a significant increase in their success. These results highlight the importance of the specific concentration of FRF employed when designing experiments, and encountered by ejaculates in nature, confirming a concentration-dependence of the effects exerted by FRF on ejaculates.

Ultimately, we conclude that FRF, under this competitive dynamic involving the co-occurrence of alternative male tactics’ seminal fluids, enables females to discriminate among territorial and sneaker ejaculates, effectively interfering with the ejaculate competition process, and to favour territorial males, thereby reinforcing females pre-mating preferences.

Notably, in a preliminary study where we tested the effect of FRF on the sperm performance of male ARTs of the grass goby, we did not find an intrinsically differential effect of any specific FRF concentration on the sperm velocity or motility of the two tactics (Pinzoni *et al.,* submitted). In that study we assessed sperm performance of individual males in presence only of their own seminal fluid, which possibly explains why we did not observe any statistical interaction between the increasing FRF concentrations and the male reproductive tactic.

Furthermore, in this species there is no significant difference in the starting sperm performance of territorial and sneaker males (measured in water and in own seminal fluid), but a difference in sperm velocity arises in presence of a mixture of the seminal fluids produced by males of the two tactics (Locatello *et al.,* 2013). Overall, the present findings, in light of the previous results, suggest that it is precisely through the interaction of all the reproductive fluids present during competitive fertilizations (seminal fluid of territorial and sneaker males + FRF) that females can differentiate between sneaker and territorial ejaculates, and bias fertilization towards their preferred phenotype, attenuating the detrimental effect of sneaker seminal fluid on territorial sperm.

The observed effect is likely mediated by molecular interactions between the female and male fluids, that will certainly need further investigation. Very little is known so far about the potential mechanisms underlying FRF-mediated paternity biases, even though some FRF-specific proteins (not found in the serum), like many glycoproteins, have been identified (Lahnsteiner *et al.,* 1995; Aguilar & Reyley, 2005; Baer *et al.,* 2009), indicating the potential for a specific function in the interaction of FRF with ejaculates (Gasparini *et al.,* 2020). Specific protein components of the FRF that could putatively be responsible for the interaction with seminal fluid have been proposed by studies of proteomic analysis and comparison of evolution rates (Swanson *et al.,* 2002; Findlay *et al.,* 2014; Sirot *et al.,* 2014; McDonough *et al.,* 2016), but only in relation to the female reproductive tract in internal fertilizers (e.g. the mammalian oviductal glycoproteins, Swanson *et al.,* 2001).

Not much more is known about the components of sneaker seminal fluid determining the impairment of territorial sperm competitiveness, with which FRF could potentially interact to compensate for this detrimental effect. Only a few studies have investigated the seminal fluid composition in relation to sperm competition in fish, suggesting that proteins with a molecular weight less than 50 kDa, as well as monosaccharides and triglycerides, could affect sperm velocity and viability (Rasotto *et al.,* 2002; Lahnsteiner *et al.,* 2004).

Regardless of the molecular mechanism regulating the interaction between the reproductive fluids, our results highlight the importance of integrating both male and female evolutionary perspectives of ejaculate competition, as well as non-gametic components, that are emerging as pivotal players of the post-mating dynamics of sexual selection.

The peculiar dynamic of FRF interference with the ejaculate competition system uncovered here suggests an incredible potential for studying the interaction between female and male reproductive fluids, that might likely envelop important mechanisms of post-mating sexual selection and sexual conflict.

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Ethics

The experiments conducted herein comply with the current Italian laws (permission OPBA University of Padova no 39/2021).

Author contributions

All authors conceived the study, P.L., L.L. and R.M.B. designed the experiments, P.L. and L.L. developed the methods. P.L. collected and analysed the data and wrote the original draft. All authors reviewed and edited the draft and contributed to the final version of the manuscript.

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6. CLOSING REMARKS

General discussion

In this thesis, I undertook a detailed examination of the role of FRF in the post-mating dynamics of sexual selection, addressing some of the outstanding issues regarding the proximate mechanisms and evolutionary consequences of such processes. This objective was addressed by employing two different species with external fertilization, the zebrafish (*Danio rerio*) and the grass goby (*Zosterisessor ophiocephalus*), characterized by radically different reproductive systems and sexual selective pressures, in order to get a broad picture of the female evolutionary interests at the post-mating level.

In the zebrafish, the research was focused on the proximate mechanisms of FRF selective role, with the goal of deepening the female perspective of the sperm competition game by exploring purely female effects, mediated by the FRF effects on the eggs, in paper I, and effects arising from the interaction of FRF with ejaculates, in paper II. The experiments presented in paper I uncover a novel mechanism of female post-mating control by which females can increase the opportunities for post-mating sexual selection, and ultimately for selecting the best partner, mediated by FRF, and acting on the fertilization window of the eggs. In presence of the FRF, indeed, the egg fertilization window is prolonged, providing externally fertilizing females with the possibility of mating polyandrously, and thus to increase the occurrence and degree of multiple paternity in their offspring. This constitutes the first evidence of an effect of FRF extending into post-mating sexual selection that acts on the eggs rather than on the sperm and broadens our knowledge of the mechanisms by which females can affect these selective processes. It has yet to be tested whether this mechanism could have adaptive benefits for females in terms of offspring quality, but I believe that further research on this topic could offer valuable insights.

In light of these results showing the potential of FRF to increase the opportunities for sperm competition, an influence of FRF over the outcome of competitive fertilizations, possibly affecting both sperm competitiveness and fertilization success of rival males, can be expected. I explored this hypothesis in paper II, investigating whether, and to what extent, females can control the outcome of competitive fertilizations via FRF, directly linking for the first time the phenotypic effects of FRF on male post-mating traits with fertilization biases. Specifically, FRF was shown to influence fertilization dynamics by acting on sperm swimming velocity, that, in turn, emerged as the main predictor of male post-mating success for this species.

Understanding the mechanisms by which selection under sperm competition targets ejaculate quality traits has been identified as a key goal of post-mating sexual selection research (Snook, 2005; Pizzari & Parker, 2009; Fitzpatrik & Lupold, 2014) and the results from paper II emphasize the importance of adopting a multivariate approach considering both phenotypic and fitness effects within the same combination of individuals to tackle this issue. The findings of this study, by effectively disentangling the male from the female-driven effects, provide a key indication of how powerful female processes, here mediated by FRF, can be in rebalancing the arena for sperm competition and enabling female to bias fertilization to their advantage, even in species with external fertilization. The combined results of paper I and II suggest that FRF plays a central role in the post-mating dynamics of sexual selection in the zebrafish, mediating a complex interplay of multiple effects, that seem to work in synergy to ultimately enable females to favour the most suitable partner.

With the grass goby (*Zosterisessor ophiocephalus*), because of its peculiar male mating system with distinct, easily identifiable, male phenotypes, I explored the potential for FRF-mediated directional processes of cryptic female choice, towards a specific male phenotype that is known to be preferred by the females at the pre-mating level (i.e., the territorial phenotype).

The results from paper III revealed that FRF does not have an intrinsically differential effect over ejaculates of territorial and sneaker males of the grass goby, and that the sperm performance in presence of FRF depends on specific male-female interactions. This suggests that different females might favour different male phenotypes at the post-mating level, perhaps depending on their status/quality, an intriguing possibility that could inspire further investigation. Nevertheless, the experiments of paper III revealed a differential concentration-dependent effect of FRF over sneaker and territorial sperm performance. Through the gradient of fluid concentration that naturally forms in the water, females seem, indeed, to favour sperm depending on their proximity to the eggs during the spawning event. Such a process typically favours territorial males (and thereby reinforces females pre-mating preferences), as they always release sperm in close proximity to the eggs, but can likewise advantage sperm of those sneakers that are able to get closer, allowing flexibility in the direction of female post-mating choice. Overall, such a cryptic choice, dependent on FRF concentration, appears to mediate a directional selection, not acting on a specific male phenotype but rather on male ability to get the most favourable position to fertilize the eggs. This finding enhances our understanding of the complexities of post-mating sexual selection in external fertilizers, offering an important example of how male and female pre-mating behaviours can have repercussions on the post-mating gametic interactions.

The concentration-dependence of the effects exerted by FRF, shown in both paper III and IV, evidences the importance of experimentally simulating, as much as possible, the spawning conditions encountered by individuals and gametes. In fact, the demonstration of purely directional processes of post-mating sexual selection in external fertilizers has proven challenging and rather complex (Alonzo *et al,* 2016; Lenhert *et al.,* 2017), possibly because of several potential factors that are still being neglected in this context. Among these factors, I hypothesized that the interaction of FRF with male seminal fluids, that is a natural occurrence in all reproductive events, may significantly impact the fertilization success of rival males, particularly in the context of ARTs. In paper IV, indeed, a directional process of post-mating control was uncovered, reinforcing the pre-mating preference of grass goby females for territorial males, mediated by the interaction of these two reproductive fluids. Acting on the tactic-specific effect of seminal fluid on the sperm performance of the opposite tactic, FRF seems to allow females to discriminate among ejaculates of the different male phenotypes and control the sperm competition outcome, possibly through a molecular inter-communication between these fluids that will certainly need further investigation. Regardless of the specific mechanism behind this, paper IV demonstrates that FRF is able to mediate a directional mate choice at the post-mating level, by rebalancing the ejaculates competition to mitigate the detrimental effect that sneaker seminal fluid plays on territorials' sperm performance. This constitutes the first evidence of a potential sexual conflict carried on by the reproductive fluids of the two sexes, and advocates for an increased consideration of male and female non-gametic components in the context of sexual selection.

Together, the role of FRF concentration and interaction with seminal fluid uncovered thanks to the grass goby, represent an important advance in our understanding of the gamete level mechanisms driving fertilizations biases, raising the question of whether these factors might affect sperm competition outcomes in a broader array of taxa.

Conclusions

The overall findings collected in this thesis ultimately provide a key indication of the central role of FRF in post-mating sexual selection, with evidence of a powerful female-driven interference over the ejaculate competition system. In both species indeed, important effects of FRF on the fertilization process were observed, as well as overall patterns of selection on ejaculate traits across reproductive scenarios. The comparison of the effects uncovered in these different species also suggests that the cryptic choice mediated by FRF could be “tailored” to the female reproductive needs at the inter-specific level, and convey both directional and non-directional selection, seemingly in response to different patterns of female pre-mating control. These findings add to the growing body of evidence that FRF might play a much more complex role than anticipated in the post-mating dynamics of sexual selection (Alonzo *et al.,* 2016; Lymbery *et al.,* 2017; Gasparini *et al.,* 2020;), evidencing how the female environment must be taken into account when studying dynamics of ejaculates competition.

The findings of my PhD research ultimately contribute to the resolution of different open questions in the field, while also generating new avenues for future investigation. As previously noted in the discussions of the different papers, it may be worthwhile to further investigate the role of the female identity and status/quality, to potentially uncover previously unexplored aspects of female allocation. Additionally, the exploration of the adaptive outcomes of both eggs and sperm-mediated effects of FRF could potentially yield valuable insights, especially in the context of the potential genetic compatibility benefits suggested in paper II. Furthermore, results of paper IV reveal the incredible potential for further research on the molecular players involved in the FRF interaction with male seminal fluid, that might hide some fundamental mechanisms of post-mating female control and sexual conflict.

Finally, given that post-mating sexual selection under external fertilization has been suggested to have influenced the whole evolutionary history of sexual reproduction (Parker 2014), mechanistic investigations in such systems could yield taxonomically broad implications.

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