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The role of female reproductive fluid in sperm competition

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Review

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The role of non-gametic components of the ejaculate (seminal fluid) in fertility and sperm competitiveness is now well established. Surprisingly, however, we know far less about female reproductive fluid (FRF) in the context of sexual selection, and insights into male–FRF interactions in the context of sperm competition have only recently emerged. Despite this limited knowledge, evidence from taxonomically diverse species has revealed insights into the effects of FRF on sperm traits that have previously been implicated in studies of sperm competition. Specifically, through the differential effects of FRF on a range of sperm traits, including chemoattraction and alterations in sperm velocity, FRF has been shown to exert positive phenotypic effects on the sperm of males that are preferred as mating partners, or those from the most compatible or genetically diverse males. Despite these tantalizing insights into the putative sexually selected functions of FRF, we largely lack a mechanistic understanding of these processes. Taken together, the evidence presented here highlights the likely ubiquity of FRF-regulated biases in fertilization success across a diverse range of taxa, thus potentially elevating the importance of FRF to other non-gametic components that have so far been studied largely in males.

This article is part of the theme issue 'Fifty years of sperm competition'.

1. Introduction

Sperm competition was originally conceived as an extension of male–male (intrasexual) competition, but in this case through a contest that plays out among ejaculates from rival males after mating has occurred [1]. More recently, the burgeoning field of cryptic female choice [2,3], defined as female-mediated mechanisms that bias fertilization toward the sperm of specific males, emphasizes the critical role that females play in moderating the outcome of sperm competition to suit their reproductive interests. As highlighted in this special issue, the definitions of sperm competition and cryptic female choice have now been broadened considerably to include externally fertilizing species and those with less well-studied mating systems ([4], see also [5]).

The formal definitions for sperm competition and cryptic female choice emphasize how selection acts on, and targets, sperm cells, respectively. Yet an increasing body of evidence highlights the important role that non-gametic ejaculate components (seminal fluids) play during postmating sexual selection. Seminal fluids play a role in moderating the success of sperm when they compete to fertilize eggs, or in influencing or manipulating females' mating behaviour and the way they subsequently use sperm from different males [6]. For example, in *Drosophila melanogaster*, seminal fluid proteins have effects on female postmating responses, including changes in egg production, sexual receptivity and activation of the immune system, all of which serve to bias sperm utilization patterns [7].

While research on seminal fluid continues apace [see 8,9], we know far less about the potential sexually selected roles of female reproductive fluid. For the purposes of the present review, we define female reproductive fluid (hereafter FRF) as the medium, arising from females, through which sperm must pass on

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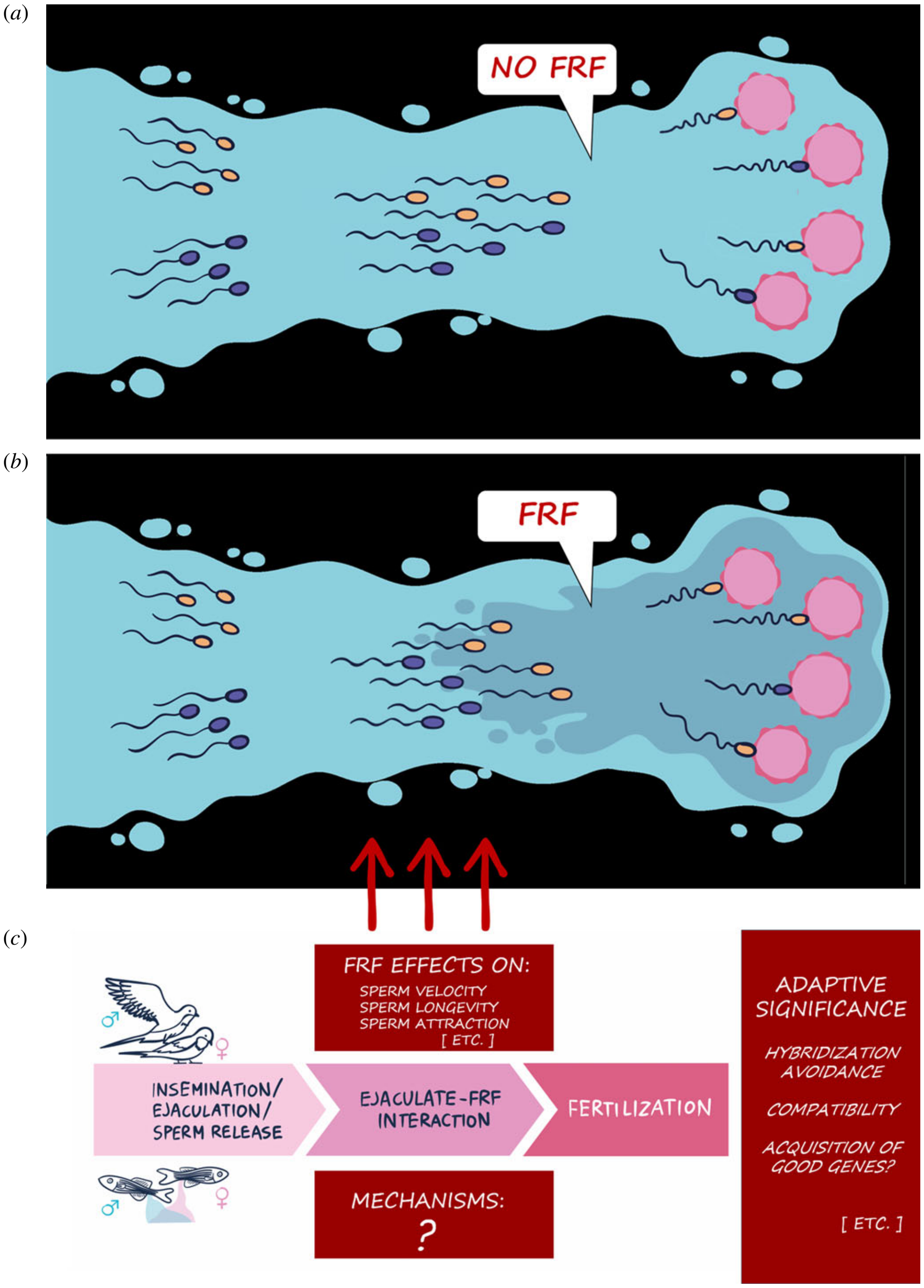

Figure 1. A schematic representation of the general mechanism by which female reproductive fluid (FRF) can affect sperm competition among ejaculates from two males (indicated by yellow and blue sperm, respectively). (*a*) Depicts a situation in which sperm competition occurs with no effect of FRF, or where FRF has been experimentally removed. Sperm are transferred/released (left side) and will fertilize eggs (right side) based on a fair raffle or their intrinsic quality. (*b*) Depicts the same situation but with FRF present. As an example, FRF differentially influences sperm velocity from the two males, increasing the velocity of the yellow sperm, which then go on to fertilize more eggs than sperm from the other male. (*c*) Summarizes the successive steps from gamete transfer/release to fertilization and highlights what we have yet to learn about this process. (Online version in colour.)

their way to fertilize eggs. In external fertilizers, the females release FRF along with the eggs; in internal fertilizers, the FRF remains inside the female reproductive tract. Components of the FRF may include (but are not limited to) ovarian, follicular, oviductal or coelomic fluid, egg chemoattractants, peripheral surfaces to eggs (e.g. egg jelly or cumulus cells), etc.

The aim of the present review is to broadly synthesize the recent literature on FRF and sexual selection, which together highlights the putative role that this fluid plays in postmating sexual selection (see figure 1 for a graphical overview of the role of FRF in postmating sexual selection). First, we briefly discuss the role that FRF plays before gametes are released, including its effect on males and eggs, and after fertilization, during the early stages of embryo development. Second, we examine the specific ways in which FRF influences sperm traits. Third, we explore the emerging evidence that FRF can play a role in moderating the selection of compatible/preferred sperm when ejaculates compete for fertilization (i.e. sexual selection). Fourth, we consider the putative mechanistic processes that underlie such sexually selected functions of FRF. Finally, we conclude by highlighting key gaps in our knowledge and provide a potential roadmap for future research in this area.

2. Effects of female reproductive fluid before gametes are released

Before considering the effects of the FRF on sperm traits, it is important to acknowledge that FRF can also affect the fertilization process independently of any effects that directly influence ejaculates. That is, FRF can have effects prior to ejaculation. In this section, we briefly consider these processes, with an emphasis on those effects that can be linked to sexual selection mechanisms and ultimately affect sperm competition.

(a) Effects on the eggs

In internal fertilizers, FRF can play a prominent role in providing the appropriate environment for fertilization and the development of oocytes (see [10] for a review on mammals). FRF can also protect eggs from oxidative stress, which might otherwise compromise embryo viability [11]. Proteomic analyses in insects and mammals showed that FRF is involved in mitigating the deleterious effects of oxidative stress [12,13], which may indicate that this is a highly conserved function of the FRF in internal fertilizers. In external fertilizers, FRF has the additional function of protecting eggs from the adverse environmental conditions into which they are released, thereby prolonging egg viability [14]. Recent work indicates that FRF is also involved in protecting eggs and embryos from pathogens. For example, proteomic analyses of FRF in species such as fishes, insects and humans confirmed the presence of proteins related to the immune system and serving a proteolytic function within the FRF [15–18]. In summary, research on the effects of FRF on female gametes has focused on naturally selected functions and predominantly those that increase egg viability. Intriguingly, some of these effects of FRF on eggs may also indirectly influence postmating sexual selection. For example, recent evidence from zebrafish (*Danio rerio*) suggests that FRF is also able to extend the fertilization window (time available for fertilization since egg activation), which may increase the opportunity for sperm competition and cryptic female choice (L Pinzoni, MB Rasotto, F Poli, C Gasparini 2020, unpublished data).

(b) Effects on males and ejaculates before spawning/ejaculation

At least two lines of evidence indicate that FRF can have a significant effect on postmating sexual selection through mechanisms that occur before ejaculates interact with FRF. First, by attracting males, FRF increases the opportunity for premating sexual selection, but also for female multiple matings, and hence for postmating sexual selection, as in the rainbow trout (*Oncorhynchus mykiss*), where males increase their upstream movement in the presence of FRF [19]. Second, by inducing an adjustment in male ejaculate investment, FRF may alter the outcome of postmating sexual selection, as in in the brown trout (*Salmo trutta*), where males exposed to FRF show increased fertilization success under both competitive and non-competitive circumstances [20] (and see [21] for a similar effect in goldfish, *Carassius auratus*). If the adjustment of ejaculate quality requires some time, females may indirectly favour males with whom they have recently associated (e.g. dominant males). FRF therefore has the potential to influence the dynamics of postmating sexual selection also through its effects prior to ejaculation.

3. Effects of female reproductive fluid on sperm traits

There is increasing evidence across a diverse range of internally and externally fertilizing taxa that FRF has multiple effects (compared to a neutral/control medium) on sperm traits typically associated with sperm competition success [22,23].

Irrespective of reproductive mode, FRF is involved at almost every stage of the sperm's journey towards the egg, from activation to fertilization. In externally fertilizing fishes, for example, the proportion of sperm that becomes motile after release is generally higher in the presence, compared to the absence, of FRF [24]. In internal fertilizers, such as insects, FRF increases the proportion of viable sperm [25,26], although this effect is not universal [27,28]. By contrast, the positive effect of FRF on sperm longevity is so far proving to be ubiquitous across taxa, including both internally and externally fertilizing fishes [29,30], birds [31], and mammals [32,33]. In mammals, FRF induces both sperm capacitation and the acrosome reaction, which are necessary precursors to fertilization [34,35].

FRF-mediated sperm chemotaxis describes the capacity of sperm to respond to chemical attractants in order to locate and swim towards the egg. Much of the evidence for FRF-mediated sperm chemoattraction comes from marine invertebrates [36], but sperm chemoattraction mediated by the FRF has also been demonstrated in a range of internally and externally fertilizing taxa, including fishes [24,37], mammals [38–40] and indeed humans [41,42], among many other metazoans [43]. Interestingly, despite the fact that sperm chemotaxis was one of the first components of the FRF–ejaculate interaction to be documented, with research on the topic dating back to the end of the nineteenth century (see electronic supplementary material), it has rarely been considered in the context of sperm competition until very recently (see §4).

Finally, sperm swimming behaviour, collectively termed 'sperm motility', is arguably the most common trait used to assess fertility and is commonly used as a proxy of sperm competitiveness in sexual selection studies [22,44]. Many studies on fishes have shown that sperm swimming speed is usually higher in the presence of FRF compared to a control solution [24,45]. The effects of FRF on parameters such as sperm velocity and sperm trajectory (e.g. linearity) have been reported also in marine invertebrates [36], amphibians [46], mammals [47] and birds ([31], but see [48]).

Overall, the evidence to date indicates that FRF can significantly alter (generally improve) sperm behaviour, including enhanced sperm capacitation, viability and longevity, chemoattraction, and sperm swimming velocity and trajectory.

4. Effects on sperm competitiveness and fertilization outcome

Here, we briefly summarize the direct evidence for FRF-mediated sexual selection from studies reporting fertilization biases when males from the same population compete to fertilize the same batch of eggs, along with evidence that FRF *differentially* affects sperm traits known to be associated with competitive fertilization success. We further illustrate the role of FRF in postmating sexual selection in species in which males exhibit alternative reproductive tactics, and finally, we briefly illustrate the potential role that FRF plays in reproductive isolation. All these studies are reported in electronic supplementary material tables 1 and 2.

(a) Differential fertilization success

Much of the evidence for FRF-mediated sexual selection suggests that these mechanisms serve to bias fertilization toward the sperm of the most compatible partner. A study of internally fertilizing guppies (*Poecilia reticulata*) revealed that FRF functions as an inbreeding avoidance mechanism, increasing the velocity of sperm from unrelated males, ultimately affecting competitive fertilization success towards the unrelated males [49]. In the externally fertilizing mussel (*Mytilus galloprovincialis*), FRF differentially attracts sperm from specific males [50], and these preferences predict both sperm swimming behaviour and offspring survival [51] and ultimately determine competitive fertilization success [52]. In the chinook salmon (*Oncorhynchus tshawytscha*), sperm swimming velocity measured in the FRF has been shown to better predict competitive paternity success than sperm velocity measured in the absence of FRF [53]. Further evidence for FRF-moderated fertilization biases comes from the ocellated wrasse (*Symphodus ocellatus*), where FRF was shown to decrease the numbers advantage that would otherwise be enjoyed by sneaking males (producing more sperm), thereby increasing the relative importance of sperm velocity and favouring preferred male phenotypes during sperm competition [54].

(b) Differential effects of female reproductive fluid on sperm performance

Other studies indirectly suggest that FRF affects sperm competition, by reporting differential (male-by-female) effects of FRF on sperm performance. These studies have been performed predominantly on external fertilizers (notably fishes), including arctic charr (*Salvelinus alpinus*) [55], chinook salmon [56,57] and the zebrafish [30], but there is also evidence in internal fertilizers, including guppies (see above [49]) and humans, where FRF has recently been shown to differentially attract sperm from different males [42]. Although many of these studies implicate FRF as a critical moderator of sperm competition, not all of them have revealed consistent effects. For example, studies of the quacking frog (*Crinia georgiana*) [46], lake trout (*Salvelinus namaycush*) [58], arctic charr [59] and capelin (*Mallotus villosus*) [60] reported no evidence for male-by-female interaction effects moderated by the FRF. Similarly, in the ant (*Acromyrmex echinatior*), there is no evidence of FRF-mediated inbreeding avoidance [26].

(c) Female reproductive fluid and alternative reproductive tactics

Recent studies have explored the possibility that FRF affects sperm competition in species where males employ alternative reproductive tactics, ultimately favouring males with 'preferred' mating tactics. Studies investigating FRF-by-tactic interactions have so far focussed on fishes, where alternative male reproductive tactics are more common. In the chinook salmon (*O. tshawytscha*), for example, FRF increases the velocity of sperm from the dominant males but not those from sneakers, resulting in a paternity bias that ultimately favours dominant males [61]. Similarly, in the masu salmon (*Oncorhynchus masou*), FRF increases the velocity and motility of sperm from dominant males more than sperm from sneaky males [62]. By contrast, in the ocellated wrasse, FRF does not differentially affect the performance of sperm from alternative male phenotypes, but decreases the sperm numerical advantage that would otherwise favour sneakers in sperm competition (see above [54]).

(d) Hybridization avoidance and reproductive isolation

FRF has been shown to discriminate between conspecific and heterospecific sperm, favouring fertilization from males of the same species and thus serving as an anti-hybridization strategy (see also electronic supplementary material for early studies on sperm chemotaxis). Species-specific effects of FRF on sperm traits have been reported in echinoderms [63], molluscs [64,65], fishes [66,67] and birds [68]. Interestingly, the species-specific discrimination effect of FRF seems to be positively associated with the risk of hybridization, as it was reported in the pied and collared flycatchers (gen. *Ficedula*), which frequently hybridize [68], but this effect was not reported among bird species with low risk of hybridization [31]. Similarly, FRF differentially affects the velocity of sperm from sympatric and allopatric males of the same species, suggesting that FRF-mediated effects on sperm may be involved in reproductive isolation among populations that ultimately leads to speciation (in guppies and Atlantic cod [69,70]).

5. Female reproductive fluid–ejaculate interactions: mechanisms

The search for underlying mechanisms of the FRF's effect on postmating sexual selection has so far focused on the composition of these fluids. The chemical characteristics of FRF, such as osmolality, pH and ionic concentration (Na^+, K^+, Mg^{2+} and Ca^{2+}) are likely to be important in both external [37] and internal fertilizers [6]. However, biochemical components of FRF, such as nutrients (glucose, pyruvate, lactate and fructose), free amino acids, hormones (e.g. prostaglandins, steroids, progesterone and growth factors), and proteins also play a pivotal role [37]. In fruit flies, for example, female reproductive proteomes of sibling species express unique secreted proteins, suggesting their involvement in postmating, prezygotic reproductive isolation [71]. Interestingly, while some FRF proteins, like albumin and immunoglobulins, are also found in the serum, others are FRF-specific, like many glycoproteins [10,13,72], suggesting a specific function in FRF–ejaculate interactions. Macromolecules, like proteins, carbohydrates, lipids or female exosomes, bind to sperm and affect their performance [6,73], while sugars can prolong sperm longevity [74,75], and progesterone is a well-known chemoattractant for mammalian sperm [76]. Mechanisms underlying FRF-mediated paternity biases may also include physiological or structural modifications to sperm [6], although for the most part such functions have not been investigated. One known example of this comes from the mussel *M. galloprovincialis*, where FRF has been shown to induce modifications on the sperm surface glycans [77] and hence affect the acrosome reaction to favour specific males at fertilization [78]. Chemical and biochemical components of FRF may interact in influencing sperm performance, as FRF Ca^{2+} concentration and FRF proteins may interact to modulate the activity of Ca^{2+} sperm-specific channels such as *CatSper* and *pdk2*, ultimately affecting sperm hyper-activation [79].

FRF can also affect competitive fertilization success by interacting with seminal fluid in addition to, or instead of, the sperm. Empirical evidence comes from bees and ants, where FRF inhibits seminal fluid serine proteases found in the seminal fluid, resulting in an increase in the viability of sperm stored by the female [17,80]. Specific FRF protein components putatively involved in the interactions with seminal fluid have been identified by proteomic analysis [81,82], or by comparing their sequence evolution rate [83,84]. For example, mammalian oviductal glycoproteins show signs of positive Darwinian selection [85], suggesting that they may play a role in FRF–seminal fluid interaction [86].

The scant knowledge of FRF-mediated mechanisms means that we can only speculate about the patterns of among-individual variation in their composition. Three distinct, non-mutually exclusive scenarios may be envisaged. First, FRF composition may show little among-female variation and instead may universally favour a preferred male phenotype [54], thus facilitating directional (i.e. consistent) postmating sexual selection. Second, FRF may vary among females in the relative quantity of one or more of its components [16,70], which in turn might differentially affect male sperm competition success. In this way, selection would be non-directional, in the sense that FRF would function to select the sperm from specific males, and these preferences would not be universal across females. Such a mechanism could act in conjunction with among-male variation in sperm/ejaculate characteristics (e.g. analogous to gamete reproductive proteins in eggs and their specific sperm ligands; see [36]). For example, variability in FRF concentration of specific ions, odorants or amino acids could interact with 'taste' receptors expressed in sperm, influencing sperm traits and ultimately fertilization success [87,88]. Third, FRF can be involved in the recognition of ejaculates from genetically related males, generating patterns of disassortative postmating sexual selection [49,53]. Under this scenario, a matched variability in the protein products of hypervariable genes, such as MHC, odorant and taste receptors, should be observed in the sperm/ejaculate *and* FRF. The observed pattern of postmating sexual selection mediated by the FRF may therefore help us to identify the mechanisms underlying FRF–ejaculate interactions.

6. Conclusion and future directions

Our review highlights the putative sexually selected functions of female reproductive fluid, which have only recently emerged in the literature. In the same way that research on cryptic female choice lagged a long way behind sperm competition, research on FRF has lagged a long way behind that of seminal fluid and we are only now starting to appreciate the potential for FRF to serve a role in sexual selection.

Much of the explicit evidence for sexually selected roles of FRF comes from a small handful of organisms, notably fishes [49,53,54] and mussels [52]. Thus, one of the key messages of this review is that we require greater taxonomic breadth in studies that search for sexually selected functions of FRF. Similarly, the considerable evidence from diverse taxa that FRF can promote changes in sperm traits compared to a neutral medium (§3) has only rarely been extended to evaluate the possible significance of such effects for sexual selection (§4). Some limitations can be attributed to logistics, with FRF being available only seasonally, or its amount being limited. However, in some cases, these limitations can be overcome, for example, by freezing FRF (e.g. salmon [89]), and/or using small quantities (few µl) of FRF for the assays (e.g. zebrafish [30] or ants [26]). The bias toward external fertilizers (see electronic supplementary material tables 1 and 2) is attributable to the fact that FRF is easier to collect and manipulate in external fertilizers than in internal fertilizers. However, FRF collection techniques have been developed in many internal fertilizing animals (e.g. [26,42,49,90]), making it possible to manipulate and test the effect of FRF on sperm traits in a sperm competition context. Furthermore, *in vitro* fertilization can be performed in many internal fertilizers, making it possible to extend the research on the effect of FRF on competitive fertilization to these taxa. To this end, rodents are promising candidates for further research, as *in vitro* fertilization techniques are well developed and routinely used for sexual selection studies in this group (e.g. in the mouse [91,92]). The combination of proteomic studies on FRF composition, associated with genetic manipulation, can provide another promising tool to investigate the role of FRF in postmating sexual selection in model species with internal fertilization, such as *Drosophila* [6].

Finally, our review highlights a number of possible mechanisms by which FRF may function to selectively bias fertilization towards specific males. Many such mechanisms, which have been discussed recently in terms of their putative roles in postmating sexual selection [6,93], involve FRF, but only a handful of studies have implicated it as an agent of sexual selection. Moreover, evidence that extends these studies to an understanding of the molecular and physiological processes that underlie FRF-modulated processes of sexual selection is extremely rare (e.g. [17]). Thus, we see enormous scope for future studies that employ mechanistic (e.g. molecular, proteomic and physiological) approaches to understand the fine-scale processes that enable FRF to selectively bias fertilizations. We anticipate that such mechanisms are likely to be widespread, given that many of these processes have been evolutionarily conserved during the 'cascade' (see [94]) that led to the evolutionary diversification of animals and the rise of pre- and postmating sexual selection.

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