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Evolutionary biology

Female reproductive fluid attracts more and better sperm: implications for within-ejaculate cryptic female choice

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Mounting evidence shows that the female reproductive fluid (FRF) can differently affect sperm performance of different males by biasing paternity share among competing males. Here, we tested for the first time the potential of 'within-ejaculate cryptic female choice' mediated by the FRF in the zebrafish (*Danio rerio*). Using a recently developed sperm selection chamber, we separated and collected FRF-selected from non-selected sperm to compare the two subpopulations of sperm in terms of sperm number, viability, DNA integrity and fertilizing ability. We showed that the sperm attracted by FRF are more numerous, more viable and with higher DNA integrity. In addition, FRF-selected sperm fertilized more eggs, but if this is due to fertilization ability *per se* or numerical advantage remains to be tested. Our results suggest that FRF can select sperm with a better phenotype, highlighting the crucial and impactful role that FRF might play in the process of fertilization and post-mating sexual selection dynamics, along with the potential implications for sperm selection in assisted reproductive techniques.

1. Introduction

Female reproductive fluid (FRF) has been the focus of recent scientific attention due to its role as a cryptic female choice (CFC) mechanism [1]. The FRF is released along with the eggs in external fertilizers, and in internal fertilizers is present in the female reproductive tract and around the eggs [1]. Recently, the FRF has been investigated for its potential in sperm selection and as a mediator of CFC in both internal and external fertilizer species [1]. Evidence supporting the differential effect of FRF on sperm has accumulated in recent years across different taxa, showing that the FRF can bias fertilization toward one male over another through its differential effect on sperm behaviour, including chemoattraction and performance [2–4].

If FRF can differentially affect sperm from different males, it could, in principle, be able to differentially affect sperm also within the same ejaculate. Phenotypic variation among sibling sperm within the same ejaculate has been reported [5], paving the way for the intriguing possibility of intra-ejaculate selection. The possible selection of different sperm within the same ejaculate sets sexual selection to a new, intra-individual, level: *within-ejaculate CFC* linked to *within-ejaculate sperm competition* [5,6]. Within-ejaculate CFC can favour (i) good sperm, if it acts as a filter against bad sperm (e.g., morphologically defecting, with low performance, or with compromised DNA) or (ii) compatible sperm, thus those carrying specific alleles or haplotypes, more compatible with the eggs/female.

Here, using a recently developed device for sperm selection, we test for the first time the occurrence of within-ejaculate CFC. We focus on whether FRF can mediate within-ejaculate CFC by selecting and favouring phenotypically good

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sperm. To do so, we use the zebrafish (*Danio rerio*) in which the role of FRF in fertilization and post-mating sexual selection has already been established [7–9]. The sperm selection chamber allows separation and collection of sperm attracted by the FRF (developed by [8]), making it possible to compare two subpopulations of motile sperm within the same ejaculate (and the same trial) in terms of sperm number, sperm quality (viability) and DNA integrity. Finally, in a separate experiment, we also compared the fertilization ability of FRF-selected and non-selected sperm.

2. Materials and methods

(a) Fish maintenance

Zebrafish used in this study were Tübingen wild-type, raised and maintained at the Zebrafish facility (University of Padova) under standard laboratory conditions (12 : 12 light–dark cycle; water temperature 28 ± 1°C). The fish (aged 6–9 months) were housed in equal sex ratio and density (12 individuals/tank) in 3.5 l tanks placed in a recirculating system (Tecniplast). Fish were fed *ad libitum* twice a day with dry food (TetraMin) and live brine shrimp nauplii (*Artemia salina*).

(b) Gametes and female reproductive fluid collection

Ejaculate and FRF collection were performed following established protocols (described in [7,10]). The FRF was collected with a 3 µl micropipette (Drummond), transferred to an Eppendorf tube and diluted to final concentration of 10% in water. The tube with the diluted FRF was kept on ice until use (within 30 min).

(i) Experiment 1 sperm phenotype

In each trial, FRF and sperm from one female and one male were added to the sperm selection chamber, following the protocol described in [8] and depicted in figure 1. The device separates sperm into FRF-selected (retrieved from the FRF well) and non-selected sperm (retrieved from the control well, filled with freshwater). Immediately after collection, we assessed (i) sperm number, (ii) sperm viability and (iii) sperm DNA integrity. We used a total of 20 unique male–female combinations, using 20 females and 19 males (one male was used twice). (i) Sperm number and (ii) sperm viability were estimated using a Luna-FL Dual Fluorescence Cell Counter (Logos Biosystems, Korea) following the protocol described in [10]. Sperm viability was assessed on 589.5 ± 71.5 s.e. sperm per sample. (iii) Sperm DNA integrity was estimated by counting the sperm that showed fragmented (single- and double-strand breaks) or not fragmented DNA according to the sperm chromatin dispersion technique using the Halomax-SCD kit (Halotech DNA), following the manufacturer's instructions (the protocol is reported in full in [10]). Sperm DNA integrity was assessed on 78.2 ± 12.3 s.e. sperm per sample. The assessment of sperm number and phenotype was performed blind to the treatment.

(ii) Experiment 2 fertilization rate

Once FRF was collected from the eggs, the remaining FRF was rinsed with a 0.5% solution of BSA (pH 8), and left in this solution to keep the eggs inactivated [11] until use (within 30 min from collection). From each female, eggs were divided into two batches (mean: 28.6 ± 2.1 s.e.) to perform IVFs. All IVFs were performed on eggs without FRF to avoid confounding the effects of FRF selection with those on the eggs [9]. FRF-selected and non-selected sperm collected from the sperm selection chamber as described above (using the FRF from the eggs used for the fertilization) were added to either one or the other batch (in random order) and left undisturbed for 3 min. After fertilization, the eggs were transferred to a Petri dish and placed in an incubator at 28°C. Fertilization rate was assessed 1 h after fertilization (fertilization rate in IVFs has been previously demonstrated to be repeatable; see [9]). For this experiment, we used 12 unique pairs of male–female (different from those used in experiment 1). All procedures were carried out on a heated pad kept at 28°C to ensure a constant and optimal temperature for fertilization.

(c) Statistical analysis

Data analyses were performed using R v. 4.2.0 [12]. We tested sperm number, sperm viability, sperm DNA integrity and fertilization rate fitting a generalized mixed model with the 'glmer' function ('lme4' package [13]). For viability, DNA fragmentation, and fertilization rate, we assumed a binomial error distribution and a logit link function. In these models, the response variable was modelled as number of successes and number of failures using the 'cbind' function. Sperm number was tested with a γ-distribution and a log link function. We fitted the models with the treatment (FRF-selected versus non-selected) as a fixed effect and pair ID as a random factor. Every model complied with the assumptions. In particular, we checked the residuals of each model by inspecting residuals versus fitted values. The sperm viability model had a possible outlier in the residual distribution, removing that data point improved the distribution of the residuals without changing the results. As the model on sperm viability was overdispersed (4.06), it was corrected for overdispersion by adding an observation-level random effect. We calculated *p*-values of the fixed effect using Type II Wald chi-square tests using the 'Anova' function in the 'car' package [14]. Effect sizes (Hedges' *g*) and the associated 95% CIs were calculated using the 'effectsize' package [15]. Means and s.e. are reported.

3. Results

(a) Experiment 1 sperm phenotype

(i) Sperm number. The number of sperm retrieved from the FRF well was significantly higher than the number of sperm collected from the control well (table 1, figure 2*a*). FRF-selected sperm were on average twice the number of non-selected sperm (934.65 ± 149.99 versus 535.05 ± 70.52, respectively), with an average within-pairs difference of 101% (± 28.8).

(ii) Sperm viability. FRF-selected sperm were significantly more viable than non-selected sperm (table 1, figure 2*b*). On average, the proportion of live sperm over the total was 0.66 ± 0.04 for the FRF-selected sperm and 0.42 ± 0.05 for the non-selected sperm. The average within-pairs difference between FRF-selected and non-selected sperm was 107% (± 41.5).

(iii) Sperm DNA integrity. There was a higher proportion of sperm with fragmented DNA in the non-selected sperm group (table 1, figure 2*c*). The proportion of sperm with intact DNA was 0.24 ± 0.03 for the FRF-selected sperm and 0.15 ± 0.03 for the non-selected sperm, with an average within-pairs difference of 131% (± 48.9).

(b) Experiment 2 fertilization rate

Fertilization rate was higher when using FRF-selected sperm than when non-selected sperm were used (33 ± 4.4% versus

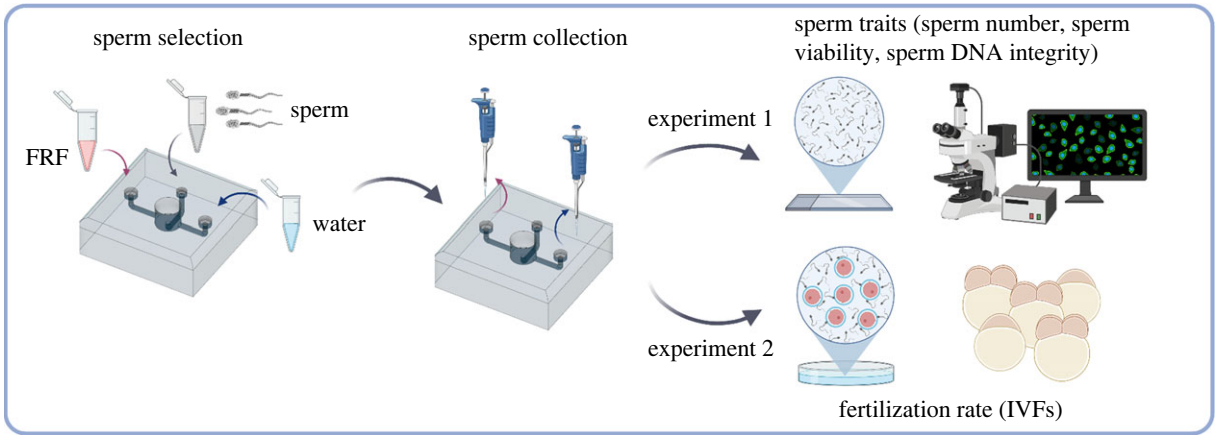

Figure 1. Schematic view of the experimental design used in this study. First step is to add sperm into the central well of the sperm selection chamber. After 20 s sperm are retrieved from both wells (one filled with 10% FRF, the other with freshwater as a control). Sperm are then used for the assessment of sperm number and sperm quality (experiment 1) and for fertilization success (experiment 2).

Table 1. Results from the mixed models on the within-ejaculate sperm traits comparison between FRF-selected sperm and non-selected sperm. Sperm number model is fitted with γ-distribution, sperm viability and DNA integrity models are fitted with binomial distribution. More details on the models used are reported in the main text.

	treatment (fixed factor)			ID (random intercept)	intercept			effect size
trait	estimates ± s.e.	χ^2	*p*	variance ± s.d.	estimates ± s.e.	statistic	*p*	Hedges' *g* (95% CIs)
number of sperm	0.52 ± 0.11	20.927	<0.001	0.31 ± 0.56	6.01 ± 0.20	29.563	< 0.001	0.75 (0.11, 1.37)
sperm viability	1.09 ± 0.18	33.990	<0.001	0.40 ± 0.63	−0.35 ± 0.19	−1.802	0.072	1.26 (0.58, 1.92)
sperm DNA integrity	0.60 ± 0.13	21.473	<0.001	0.51 ± 0.71	2.01 ± 0.19	10.863	< 0.001	0.76 (0.11, 1.40)
fertilization rate	−0.42 ± 0.17	6.034	0.014	0.50 ± 0.71	−0.73 ± 0.24	−3.023	0.003	0.33 (−0.45, 1.10)

27 ± 5.2%, respectively; $\chi^2 = 6.034$, $p = 0.014$). The average within-pairs difference in percentage was 37.4% (± 32.7).

4. Discussion

In most species, sperm are produced in excess compared to the number of eggs, and even in the absence of competition with sperm from rival males, there is a strong selection; only a few of them will come closer to the eggs at fertilization and only one will be able to ultimately fuse with the egg cell. The competition among sperm to fertilize the eggs is affected by the microenvironment in which they swim and move in before encountering the eggs, and in both internal and external fertilizers this environment is deeply interconnected with the FRF. The role of FRF in sperm selection across different males has been recently demonstrated in many species (reviewed in [1]), but so far no studies have investigated its role in sperm selection within the same ejaculate (within-ejaculate CFC). Our results provide evidence that sperm selected by the FRF are more numerous, more viable, and with higher DNA integrity. Also, the fertilization rate is significantly higher with sperm that are FRF-selected compared to the fertilization rate with non-selected sperm, albeit this difference can be due to the numerical advantage of FRF-selected sperm.

The differences in sperm phenotype we found are likely related to their genotype, corroborating the hypothesis that the FRF may act as a mediator of within-ejaculate CFC: the presence of FRF in the medium allows better sperm to come closer to the eggs. In particular, FRF-selected sperm had higher viability and higher DNA integrity. High sperm viability as we have measured it (after 20 s), is a cross-sectional measure of sperm longevity, and within-ejaculate differences in sperm longevity have been linked to increased early and late offspring fitness in this species [16,17]. Furthermore, in zebrafish, sperm with fragmented DNA may retain the ability to fertilize but produce low-quality embryos, with less chances of developing successfully [18]. The comparison between FRF-selected and non-selected sperm resulted in a large effect size (>0.75, see table 1) for all the traits considered indicating that the differences are not only statistically significant but also biologically important.

We ascribe the differences in sperm traits to FRF selection, but we are also aware that these effects may also be due to a 'boost' effect (hereafter, a generalized positive effect on sperm

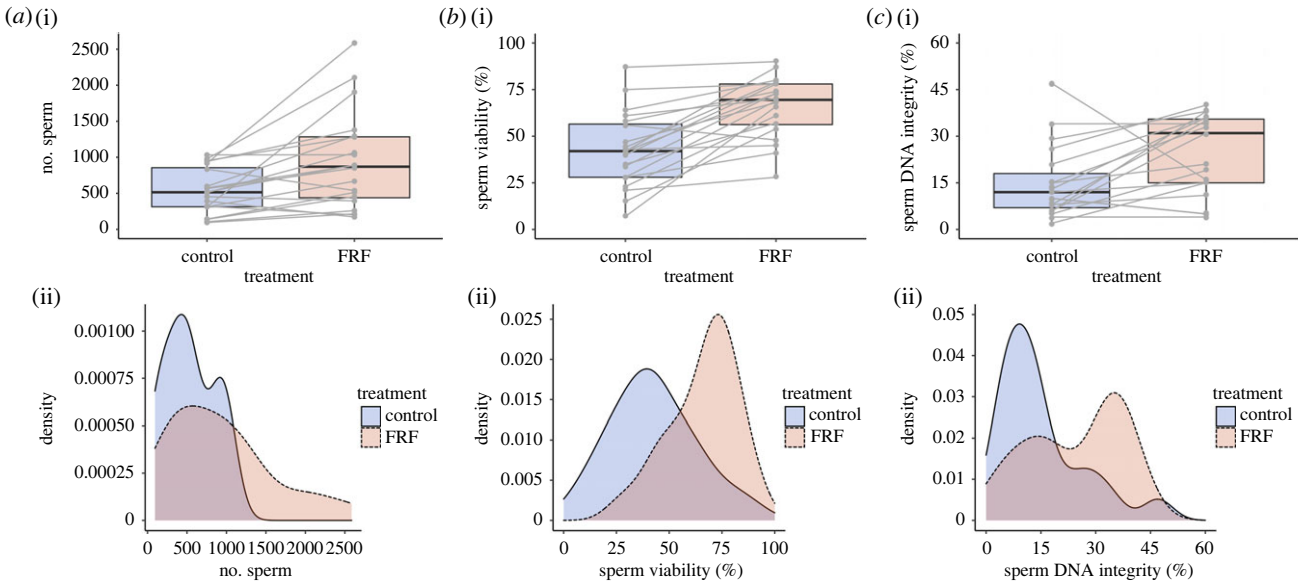

Figure 2. Box plot depicting differences between FRF-selected and non-selected sperm within the same ejaculate. (*a*) Number of sperm, (*b*) sperm viability and (*c*) sperm DNA integrity. (i) Box plots with data points, the lines connect FRF-selected and non-selected sperm within the same ejaculate; (ii) density curves (solid line: control, dashed line: FRF). Percentages are used only for the ease of visualization.

traits) of the FRF. Indeed, FRF has a beneficial effect on sperm (increased motility, [7]) in this species. Nevertheless, our results, together with the previous evidence available, suggest that the contribution of the boost effect may be minor compared to the FRF selection itself. First, the concentration of FRF used in this study is less than that used in previous studies (10% versus 20%) [7], and if the effect of FRF on sperm traits is dose dependent (that remains to be tested), the boost effect on sperm traits may be reduced. Second, in this species, the beneficial effect of FRF on sperm performance became evident only after a certain time (30 s) since the contact between the sperm and the FRF [7], while in this study, we collected sperm from the chamber 20 s after activation, as in [8], thus before the effect on sperm chemokinesis is usually observed. Third, in the sperm selection chamber, before being collected for phenotyping, the sperm were not incubated in FRF as in the study mentioned above but were rather attracted by a gradient of it. Therefore, both the time and the average FRF concentration in our sperm selection chamber were lower than in previous experiments. Taken together, these considerations lessen the possibility that the differences in the sperm phenotype we found were due to the boost effect of FRF. The boost effect will probably contribute to exacerbate the differences between FRF-selected and non-selected sperm, thus reinforcing the results and fitness effect of the within-ejaculate CFC. Our results also indicate that FRF-selected sperm have better fertilization ability compared to non-selected sperm, despite the magnitude of this effect being marginal (as indicated by a low effect size compared to the effect on sperm traits). Moreover, the higher number of FRF-selected sperm (due to the chemoattractive ability of FRF, results of this study and [8]) can possibly alone, or in synergy, explain the increased fertilization ability.

In conclusion, our study provides the first evidence that FRF can mediate within-ejaculate CFC, attracting more sperm with a better phenotype, under a 'good sperm' scenario of adaptive CFC. Whether the FRF can also act as a selector of more compatible sperm is the next step of investigation. If corroborated in other species, the implications of our findings are important. As it may bias fertilization towards a certain sperm phenotype, within-ejaculate CFC may have important evolutionary consequences for male and female fitness, as well as for population demography. Whether the differences between FRF-selected and non-selected sperm translate into an enhanced fitness for the female through increased fertility or offspring quality remains to be investigated in full. Our results on the fertilization rate already suggest a beneficial direct effect of CFC in terms of increased fertility. Moreover, one of the biggest limitations of assisted reproductive techniques used in the management of human fertility and important ecological and economical species is precisely the impossibility of assessing the level of DNA integrity in sperm that will be used. The result we found in terms of improved DNA integrity in FRF-selected sperm opens an exciting avenue for managing this factor, which is possibly one of the main factors reducing successful outcomes in assisted reproductive techniques. Most prevalent sperm selection techniques (e.g. swim-up technique) are aimed at selecting sperm motility and viability, while a protocol based on FRF-mediated selection could—if these findings will be confirmed in other species—help select sperm with both increased motility and DNA integrity. We are certain that the results we presented here shed new light on the function of FRF and its role in post-mating sexual selection, and specifically on the possibility of within-ejaculate CFC.

Ethics. This study was approved by the Ethics Committee of the University of Padova (protocol number: 100/2019).

Data accessibility. The data and R code used in this study are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.crjdfn38v [19].

Authors' contributions. S.C.: conceptualization, formal analysis, methodology and writing—review and editing; A.D.: methodology and writing—review and editing; M.S.: methodology and writing—review and editing; C.G.: conceptualization, project administration, supervision and writing—original draft.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. The authors have declared no competing interests.

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