Effects of ovarian fluid on sperm traits and its implications for cryptic female choice in zebrafish

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RUNNING TITLE

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ABSTRACT

In polyandrous mating systems, females maintain the opportunity to bias male fertilization success after mating in a process known as cryptic female choice. Mechanisms of cryptic female choice have been described both in internal and external fertilizers, and may affect fertilization processes at different stages before, during and after fertilization. While in internal fertilizers, females have substantial control over sperm storage and fertilization, in external fertilizers, female control is limited. A key factor proposed to mediate cryptic female choice is the fluid surrounding the eggs, the ovarian fluid, as it may directly affect sperm performance. Here, we studied the role of ovarian fluid in post-mating sexual selection using the zebrafish, *Danio rerio*. In a first step, we assessed how ovarian fluid affects sperm swimming performance compared to freshwater. We focussed on sperm motility, velocity, swimming trajectory and longevity, all traits associated with competitive fertilization success in externally fertilizing fish. In a second step, we used a North Carolina II design to explore female, male, and female x male effects by testing sperm motility of two males in the ovarian fluid of two females in a total of eleven blocks. Our results suggest that the ovarian fluid affects sperm performance differently from freshwater. Specifically, sperm velocity, motility and longevity were higher in the ovarian fluid than in freshwater, whereas sperm linearity and beat cross frequency showed the opposite pattern. Moreover, these effects varied according to male and female identities, supporting the potential for cryptic female choice mediated by ovarian fluid in this species.

INTRODUCTION

Females mating with multiple males during a single reproductive cycle allows for sexual selection to continue after copulation in the form of post-mating (or post-ejaculatory) sexual selection (Birkhead and Pizzari 2002; Parker 2014). Theoretical studies suggest that this process is particularly intense in external fertilizers due to the co-occurrence of gametes of multiple individuals and is further exacerbated by the reduced opportunities for mate choice and/or the exclusion of rivals prior to gamete release (Birkhead and Møller 1998; Levitan 2010). Compelling evidence for evolutionary processes operating at the gamete level has fuelled studies on understanding mechanisms of post-mating processes such as sperm competition, where sperm of two or more males compete to fertilize the same set of eggs (Parker 1970). Sperm competition has largely been associated with increased investment in sperm production at interspecific and intraspecific levels (Birkhead and Møller 1998; Parker and Pizzari 2010). In more recent years, the role of sperm quality traits in determining fertilization success has been an increasingly recognised in a number of species (Firman and Simmons 2015; Simmons and Fitzpatrick 2012; Snook 2005). In particular, sperm competition favours the evolution of sperm quality traits that maximise sperm ability to outcompete rival ejaculates (Birkhead and Møller 1998; Fitzpatrick and Lüpold 2014; Pizzari and Parker 2009). Those traits include sperm velocity, sperm viability (the proportion of motile sperm in the ejaculate), sperm morphology, and sperm longevity. In particular, higher sperm velocity and sperm viability have been associated with increased sperm competition success in many species with both, internal and external fertilization (Simmons and Fitzpatrick 2012; Snook 2005). While the outcome of sperm competition has traditionally been associated with the intrinsic characteristics of competing ejaculates,

fertilization success also critically depends on post-mating interactions between males and females, and the ability of females to bias the outcome of sperm competition via mechanisms of cryptic female choice (Birkhead et al. 1993; Eberhard 1996; Thornhill 1983). Due to the challenges associated with studying these cryptic mechanisms and with effectively disentangling processes of cryptic female choice from those of sperm competition (Firman et al. 2017), we still know surprisingly little about the proximate mechanisms involved.

Females employ a variety of mechanisms to select among sperm from different males (Birkhead et al. 1993). In internal fertilizers, these mechanisms include directional sperm ejection, control of copulation duration, changes in sperm swimming behaviour during sperm migration along the reproductive tract influenced by female reproductive fluids, and selective sperm storage (Pizzari and Birkhead 2000; Peretti and Eberhard 2010; Holman and Snook 2008; Friesen et al. 2016). In external fertilizers, the fluid surrounding the eggs, namely the ovarian fluid, has been suggested to be a possible mediator of sperm selection (e.g. Urbach et al. 2005; Rosengrave et al. 2016). Ovarian fluid has been shown to affect various sperm traits such as sperm activation, chemotaxis, longevity, swimming performance and trajectory across internally and externally fertilizing taxa (Bernasconi et al. 2002; Turner and Montgomerie 2002; Urbach et al. 2005; Elofsson et al. 2006; Oliveira et al. 1999; Rosengrave et al. 2009a; Rosengrave et al. 2009b; Gasparini et al. 2012; Gasparini and Evans 2013; Liberti et al. 2016). Interestingly, the ovarian fluid of one female does not seem to affect sperm of all males in the same way, but varies depending on male and female identity, indicating cryptic female choice (Firman et al. 2017). In the internally fertilizing guppy *Poecilia reticulata* for example, ovarian fluid has been shown to increase the velocity of sperm from unrelated males and by that favouring those males during fertilization over

related male as means of inbreeding avoidance (Gasparini and Pilastro 2011). In the externally fertilizing mussel, *Mytilus galloprovincialis*, chemoattractants in the ovarian fluid were proven to select for more genetically compatible ejaculates by differentially affecting sperm performance of competing males, which resulted in higher embryonic viability and offspring survival (Oliver and Evans 2014). Similar effects have been reported in studies in externally fertilizing fish species. In the Arctic charr, *Salvelinus alpinus*, for example, Urbach et al. (2005) found that the effect of ovarian fluid on sperm velocity varied across the different male-female crosses. Furthermore, in the Chinook salmon, *Oncorhynchus tshawytscha*, ovarian fluid differentially affected sperm swimming speed, which was positively correlated with embryo survival (Rosengrave et al. 2008; 2016). Similarly, in the ocellated wrasse, *Symphodus ocellatus,* the presence of ovarian fluid increased the relative importance of sperm number over sperm velocity, which provides nesting males producing less but faster sperm with an advantage over males adopting alternative reproductive tactics producing many but slower sperm (Alonzo et al. 2016). Finally, in the Atlantic salmon, *Salmo salar*, ovarian fluid selectively enhanced chemoattraction and motility of conspecific sperm to avoid hybridization with the brown trout, *Salmo trutta*, overlapping in breeding season and grounds (Yeates et al. 2013; Alonzo et al. 2016).

In the present study, we tested the potential role of ovarian fluid in cryptic female choice in the zebrafish, *Danio rerio*, a tropical freshwater fish with external fertilization. We were interested in assessing if females can differentiate between ejaculates of different males through the effect of ovarian fluid on sperm traits. In the wild, female zebrafish ready to spawn rapidly dart multiple times into shallow water and are chased by one or multiple males (Engeszer et al. 2007; Spence et al. 2008), which often leads to multiple paternity in the resulting offspring (Paull et al. 2010; Watt et al. 2011). Post-mating sexual selection is

therefore likely to play a key role in this species. In a first step, we assessed sperm performance in a mixture of ovarian fluid and freshwater compared to pure freshwater by measuring a range of sperm motility traits and sperm longevity. In a second step, we explored whether the effect of ovarian fluid on sperm performance varies according to specific combinations of males and females. To this end, we employed a North Carolina II (NCII) design (Lynch and Walsh 1998) that allows the effective partitioning of variance in sperm performance attributable to female, male, and female x male effects (Lynch and Walsh 1998; Garcia-Gonzalez and Evans 2011).

MATERIAL AND METHODS

Fish maintenance

Zebrafish used in this experiment were AB wild-type descendants, which were raised and maintained at the Western Australian Zebrafish Experimental Research Centre (WAZERC, University of Western Australia). All fish were maintained at equal sex-ratio (10 males:10 females per tank, 3.5 L) in a recirculating rack system under standard laboratory conditions (14:10 light-dark cycle; water temperature 28 $\pm$ 1°C) and fed *ad libitum* twice per day with a mix of dry food and rotifers. Both, males and females used for the experiments were five months old. This study was approved by the Animal Ethics Committee at the University of Western Australia (approval number RA/3/100/1531).

Overview of the experimental design

To assess the effects of ovarian fluid on sperm traits compared to freshwater, we measured the swimming performance (velocity, trajectory and longevity) of sperm of 22 males in pure

138 freshwater (hereafter, 'water') and in a solution of water and ovarian fluid (see below
139 "gamete and ovarian fluid collection"). In a second set of experiments, we used a North
140 Carolina II (NCII) block design to test for female, male, and female x male effects on sperm
141 performance (Lynch and Walsh 1998). In each block, we assessed the performance of sperm
142 of two different males in the ovarian fluid of two females (2x2 full-factorial design, see
143 Figure 1) with two replicates each for a total of 11 blocks resulting in a total of 44 unique
144 combinations of male x female ovarian fluid (total of 88 replicates). The NCII design is a
145 powerful tool to disentangle female- and male-driven effects and their interaction by
146 overcoming variation due to male and female identity (Garcia-Gonzalez and Evans 2011).
147 This design has been employed to discriminate between the male and female role in
148 mechanisms of cryptic female choice and fertilization outcome in other species, including
149 for example the Chinook salmon, *O. tshawytscha* (Evans et al. 2013), the sea urchin,
150 *Heliocidaris erythrogramma* (Evans and Marshall 2005), and the mussel, *M. galloprovincialis*
151 (Lymbery et al. 2017).
152
153 Gamete and ovarian fluid collection
154 One day before the experiment, two males and two females were selected from different
155 tanks to avoid any potential effect of hierarchy (Paull et al. 2010). The selected fish were
156 transferred into small tanks (1L), where males and females were separated by a transparent
157 divider. Fish were not fed for 18 hours prior to the experiment to prevent faecal
158 contamination during gametes collection. Gametes were collected following procedures
159 described in Alavioon et al. (2017). Briefly, both males and females were first anesthetized
160 in a water bath containing MS222 (tricaine methanesulfonate, Sigma Aldrich; 0.17 g/L),
161 gently rinsed in water, and then placed under a dissecting microscope. The abdomen and

the genital area of the anaesthetised fish was carefully dried to avoid accidental activation of gametes by water. The ejaculate was collected using a glass capillary by gently squeezing the abdomen of males. The ejaculate was then diluted in 40 μL of Hank's solution, and maintained on ice until use (within one hour from collection; Hagedorn and Carter 2011; Jing et al. 2009). For ovarian fluid collection, the abdomen of the female was gently squeezed to release eggs along with the surrounding ovarian fluid onto a glass slide. The ovarian fluid was collected with a Drummond micropipette (equipped with a 34mm-diameter long plastic tip) by gently aspiring the fluid around the batch of eggs, and diluted to a final concentration of 20% in freshwater and kept on ice until use (within one hour from collection). The quantity of ovarian fluid present at spawning is difficult to estimate, as it continuously changes with time since egg release. The concentration also depends on the distance between sperm and eggs at release and on local and transient conditions of the water and ground. However, 20% dilution is likely to lie within the range of ovarian fluid concentration (between 0 to 50%) around the eggs during spawning in this species. In addition, 20% dilution was the concentration used in similar studies in other externally fertilizing fish species (e.g. Butts et al. 2012; Lehnert et al. 2016). After gamete collection, all individuals were placed in a tank with water and oxygen supply until they completely recovered. All experimental fish were used only once.

Sperm assays

For each assay, 0.5 μL of ejaculate was transferred into a well on a multi-well slide (12 well multi-test slides, MP Biomedicals), activated, and immediately covered with a coverslip. In the first part of the study, the ejaculate of a male was activated in 2.5 μL of water or of the ovarian fluid solution in random order. In the second part of the study (NCII block design),

186 the ejaculate of each male was activated in 2.5 μL of the ovarian fluid solution of two
187 different females in randomised order (see figure 1). Sperm motility was recorded through a
188 digital camera (Thorlabs, DCC3240C) connected to a phase-contrast microscope (Olympus
189 BX43). Videos were captured at 100 frames per second and analysed using ImageJ CASA
190 (Computer Assisted Sperm Analyses) plugin (Wilson-Leedy and Ingermann 2007; Purchase
191 and Earle 2012). For each sample, we assessed an average of 135.80 ± 2.52 SE sperm cell
192 tracks. The following velocity parameters were measured: curvilinear velocity (VCL, μms^{-1}),
193 trajectory (measure of path curvature: LIN, linearity), beat-cross frequency (BCF), and
194 motility (proportion of motile cells over the total). Sperm longevity was measured from the
195 videos as the time from activation until ≥ 80% of sperm in the field of view were immotile
196 (Neff et al. 2003). Sperm motility parameters were assessed every 10 seconds for the first
197 minute post activation resulting in six measurements for each sample (Wilson-Leedy and
198 Ingermann 2007). We measured each sample twice for repeatability estimates.
199
200 Statistical analyses
201 Repeatability was tested using the 'rptR' package (Gaussian or Poisson distribution was used
202 according to the error distribution of the different variables) based on 1,000 permutations.
203 Linear mixed effect models ('lmer' function of the 'lme4' package) were used to analyse
204 VCL, LIN and BCF. Sperm motility was analysed using generalised linear mixed effect models
205 ('glmer' function of the 'lme4' package) assuming a binomial error distribution and logit link
206 function. Sperm longevity was analysed using a generalised linear mixed effect model
207 ('glmer' function of the 'lme4' package) by specifying a Poisson distribution. To analyse the
208 effect of water and ovarian fluid on sperm performance, mixed effect models included
209 treatment (water and ovarian fluid) and time as fixed factors, and male ID as random factor.

Analyses performed at a single time point (e.g. at 10 seconds post activation, spa, 20 spa or 30 spa) were similar, but did not include time.

To assess the effect of male, female, and their interaction on sperm traits, mixed effect models included a fixed factor (time) and random factors (male ID, female ID and the male ID x female ID interaction term) fitted with random slopes and fixed intercept to account for the effect of time. Visual inspection of residuals in all models confirmed that the model assumptions were met. P-values for fixed effects were obtained from the F-statistic with the lmerTest package using Satterthwaite's approximation for the denominator degrees of freedom. P-values for random effects were obtained by likelihood ratio tests of the full model against the model with the specific random factor removed. All analyses were performed using R v 3.3 (R Core Team 2016).

RESULTS

Repeatability of sperm trait measurements

Repeatability was very high for all the parameters at all times in both water and ovarian fluid solution, with the exception of LIN measured at 40 spa, 50 spa and 60 spa (for repeatability estimates see Table S1 in Supplementary Materials). Sperm longevity showed significant repeatability both, in ovarian fluid solution ($R=0.479$, CI: 0.143, 0.674, $P=0.013$) and in water ($R=0.625$, CI: 0.501, 0.733, $P<0.001$).

Effect of ovarian fluid on sperm traits

VCL was significantly affected by treatment ($F_{1,742.32}$=3.93, P=0.048), by time ($F_{5,742.06}$=805.65, P<0.001) with an interaction term between treatment and time ($F_{5,742.05}$=7.73, P<0.001). As expected, VCL declined over time but at a different rate in water compared to ovarian fluid solution (Figure 2A). Sperm measured in ovarian fluid solution swam at a slower speed to begin with than sperm measured in water (at 10 spa water: 107.790 $\pm$ 36.952, ovarian fluid: 95.577 $\pm$ 19.661, $F_{1,106.53}$=25.087, P<0.001; at 20 spa water: 88.152 $\pm$ 22.364, ovarian fluid: 91.763 $\pm$ 8.029, $F_{1,108.16}$=7.823, P=0.006) but they declined at a slower rate and ended up being faster at 60 spa (water: 60.564 $\pm$ 6.367, ovarian fluid: 62.456 $\pm$ 5.020, $F_{1,101.38}$=6.609, P=0.012). LIN was significantly affected by treatment ($F_{1,742.46}$=401.34, P<0.001), by time ($F_{5,742.03}$=8.90, P<0.001), and the interaction between treatment and time was significant ($F_{5,742.02}$=10.92, P<0.001). LIN was significantly lower in ovarian fluid compared to water at all times, indicating that sperm swam in a more curvilinear trajectory in presence of ovarian fluid compared to pure water. LIN also decreased over time in ovarian fluid (between 10 spa and 30 spa) but remained constant in water (Figure 2B). Similarly, BFC was higher in water compared to ovarian fluid ($F_{1,742.12}$=335.07, P<0.001) and increased significantly over time ($F_{5,742.00}$=501.22, P<0.001) with a significant interaction between time and treatment ($F_{5,742.00}$=10.02, P<0.001) indicating that the increase in BCF over time was more rapid in water than in ovarian fluid solution (Figure 2C). The proportion of motile sperm was affected by treatment ($F_{1,753.20}$=17.87, P<0.001), time ($F_{5,753.01}$=229.35, P<0.001), and their interaction ($F_{5,753.01}$=5.167, P=0.796, P<0.001). The proportion of motile sperm was not significantly different between the two treatments at 10 spa, 20 spa or 30 spa, but was significantly higher in ovarian fluid at 40 spa (water: 0.666 $\pm$ 0.173 ovarian fluid: 0.724 $\pm$ 0.041 , $F_{1,108.13}$=10.977,Df=1, P=0.001), 50 spa (water: 0.535 $\pm$ 0.204 ovarian fluid: 0.588 $\pm$ 0.171,

257 $F_{1,108.11}$=17.067, P<0.001) and 60 spa (water: 0.393 ± 0.230 ovarian fluid: 0.456 ± 0.235,
258 $F_{1,108.14}$=21.761,P<0.001), suggesting that ovarian fluid is more successful in maintaining
259 sperm motility over time compared to water (Figure 2D). Finally, sperm longevity was higher
260 in ovarian fluid solution compared to water (ovarian fluid: 84.4 spa ± 2.13 water: 75.4 spa ±
261 2.03, X^2= 34.104, Df=1, P<0.001).
262
263 Male-by-female interaction effects on sperm traits
264 In the analyses of the outcome of the NCII breeding design (Figure 1, N=11 blocks; Table 1),
265 we found a significant effect of time on sperm velocity, linearity, BCF and motility (time
266 effect in all models: P<0.001). We also found an effect of male ID only on sperm longevity
267 (Table 1), which indicates that some males produced intrinsically longer-lived sperm than
268 other males. Most importantly, we found a significant interaction between male ID and
269 female ID for all traits considered (Table 1), indicating that the ovarian fluid of specific
270 females differentially affected sperm of specific males.
271
272 DISCUSSION
273
274 Our findings showed that ovarian fluid affects sperm motility traits differently compared to
275 freshwater alone. Sperm lived longer and swam in a more curvilinear path in ovarian fluid,
276 and their velocity slowed down over time, although less abruptly in the presence of ovarian
277 fluid compared to pure water. Moreover, the effect of ovarian fluid varied depending on
278 specific male-female combinations, which suggests that ovarian fluid may play a role in
279 mediating cryptic female choice in zebrafish through its effects on sperm performance.

Our results of an effect of ovarian fluid on sperm traits are in line with previous findings in external and internal fertilizing species (e.g. Oliveira et al. 1999; den Boer et al. 2009; Rosengrave et al. 2009a; Gasparini et al. 2012). The observed decline of sperm motility and velocity over time was expected and confirms previous observations in this species (Wilson-Leedy and Ingermann 2007) and other externally fertilizing fish (e.g. Cosson et al. 2008; Fauvel et al. 2010). Nevertheless, the patterns of sperm performance (swimming velocity and trajectory) in water and ovarian fluid over time observed in our study differ from the patterns found in the majority of the other externally fertilizing fish. Our finding, that sperm velocity during the first 30 seconds post activation was higher in water than in the ovarian fluid, is the opposite pattern of what has been described in other species (e.g. Turner and Montgomerie 2002; Rosengrave et al 2009a; Galvano et al 2013). We found no difference also in the proportion of motile sperm between the two treatments during the first 30 seconds. At 30 seconds post activation, neither sperm velocity or proportion of motile sperm no longer differed between treatments, but later than 30 seconds post activation both sperm velocity and the proportion of motile sperm decreased more rapidly in water than in the ovarian fluid. Similarly, the enhancing effects of ovarian fluid on sperm movement were found most pronounced 20-30 seconds post activation also in the Atlantic cod, *Gadus morhua* (Litvak and Trippel 1998) and in the Arctic charr, *S. alpinus* (Turner and Montgomerie 2002).

In contrast to straight line velocity, BCF increased over time both, in water and in ovarian fluid, but the increase was faster in water. Similar patterns of flagellar movement in ovarian fluid have been described in the Chinook salmon, *O. tshawytscha* (Butts et al. 2017). One possible explanation is that a higher metabolic rate associated with faster swimming speed

304 results in a shorter sperm lifespan. As BCF is related to the sperm propulsive energy, a
305 higher BCF may reflect a higher rate of energy consumption (Cosson et al. 2008; Butts et al.
306 2017) at the potential expense of longevity (Cosson 2010). The fact that we found sperm
307 longevity to be reduced in water compared to ovarian fluid further supports this idea and is
308 in line with the findings in other species (e.g. Turner and Montgomerie 2002; Elofsson et al.
309 2003; Elofsson et al. 2006; Rosengrave et al. 2009a). In addition, our results show that some
310 males produce intrinsically longer-living sperm than other males, which may reflect
311 differences in quality or condition among males. Alternatively, males may invest
312 differentially in sperm quality traits according to their role in sperm competition (Oliveira et
313 al. 2008; Taborsky and Brockmann 2010; Taborsky et al. 2018).
314 Finally, we showed that sperm trajectories were more curvilinear in ovarian fluid compared
315 to water. A more curvilinear path in the presence of ovarian fluid has also been reported in
316 the Atlantic cod, *G. morhua* (Beirao et al. 2015), and in the Pacific herring, *Clupea pallasi*
317 (Cherr et al. 2008). Interestingly, contrasting patterns have been reported in the rainbow
318 trout, *Oncorhynchus mykiss*, the lake trout, *Salvelinus namaycush*, and the Chinook salmon,
319 *O. tshawytscha,* where sperm tested in ovarian fluid exhibited a straighter path trajectory
320 than in pure water (Dietrich et al. 2008; Galvano et al. 2013; Rosengrave et al. 2009a).
321 One possible explanation for the observed differences in sperm motility in water and
322 ovarian fluid is that water may induce a reaction in fish sperm similar to a chemotactic
323 reaction described in marine invertebrate spermatozoa (Miller RL. 1985) or hyperactivation
324 in mammalian sperm (Suarez and Ho 2003). Contact with water and the resulting sperm
325 activation may translate into an initial burst of sperm velocity (VCL), coupled with an
326 increased energy demand (as indicated by the sharp increase in BCF) and subsequent
327 decrease in sperm lifespan (as indicated by both longevity and proportion of motile sperm).

328 This pattern would suggest that sperm velocity and longevity trade off against each other
329 (Levitan 2000) and perhaps this trade-off may be different in males with different
330 reproductive tactics. However, possible trade-offs between sperm traits require further
331 investigation, as a previous study in the zebrafish found no evidence for a trade-off between
332 sperm swimming velocity and longevity measured for the same sperm (Alavioon et al.
333 2017). In contrast, ovarian fluid did not cause an initial burst in sperm velocity and hence no
334 abrupt increase in BCF and decreased longevity as observed in water. Ingermann et al
335 (2011) suggested that the ionic composition of the ovarian fluid accounts for the enhanced
336 duration of sperm motility in zebrafish, consistent with findings in the freshwater sculpin,
337 *Cottus gobio* (Lahnsteiner et al. 1997) and the three-spined stickleback, *Gasterosteus*
338 *aculeatus* (Elofsson et al. 2006). The different patterns of sperm motility we found in water
339 and ovarian fluid may be adaptive and reflect the proximity of sperm to the eggs. In other
340 words, when sperm are released further away from the eggs (where the concentration of
341 ovarian fluid is low), it may be advantageous to swim faster and in a straight path to quickly
342 reach the eggs before the fertilization window runs out. But when sperm are released closer
343 to the eggs, for example when females are spawning with the preferred male, ovarian fluid
344 may adaptively prolong sperm lifespan and decrease sperm linearity to increase sperm
345 ability to find the micropyle and fertilize the eggs (Turner and Montgomerie 2002).
346 Similar variation in sperm-egg distance may be the result of males assuming different
347 spawning tactics, where sneaker males produce faster sperm, whereas dominant/territorial
348 males produce long-living sperm (Burness et al. 2004; Neff et al. 2003; Taborsky et al. 2018).
349 The pattern we found for the linearity of the swimming trajectory may also be a
350 consequence of the physical characteristics of the medium and in particular of the viscosity
351 ovarian fluid. Nevertheless, the fact that we found evidence of male x female interaction

352 also for linearity suggests that this aspect of sperm motility contributes to the fertilization
353 process. Distinguishing between the different hypotheses outlined above needs further
354 investigation. A possible next step would be to include a gradient of the ovarian fluid along
355 which to assess sperm motility to better understand and interpret the observed swimming
356 patterns.
357
358 The second important insight gained by our results is the evidence that ovarian fluid
359 differentially influences sperm of different males as indicated by the significant male-by-
360 female interaction. This finding supports the idea that the ovarian fluid may play a role in
361 cryptic female choice in this species. Ovarian fluid has been shown to mediate increased
362 genetic compatibility among partners, which in turn affected offspring survival in mussels,
363 *M. galloprovincialis* (Oliver and Evans 2014) and Chinook salmon, *O. tshawytscha*
364 (Rosengrave et al. 2016). In addition, cryptic female choice by means of the ovarian fluid
365 specifically decreases the risk of inbreeding in guppies, *P. reticulata* (Gasparini and Pilastro
366 2011), and may be a potential mechanism explaining effects on offspring survival in the lake
367 trout, *S. namaycush* (Butts et al. 2012) and in the house mice, *Mus domesticus* (Firman and
368 Simmons 2015). Genetic compatibility and inbreeding avoidance are possible evolutionary
369 forces driving cryptic female choice also in zebrafish. This hypothesis could be tested by
370 performing competitive fertilization trials among individuals with different degrees of
371 genetic compatibility. Furthermore, ovarian fluid may also differently affect sperm from
372 males with diverse social status (associated with diverse sperm traits, as in many species
373 with alternative reproductive tactics, e.g. Alonzo et al. 2016; Taborsky et al. 2018). In
374 zebrafish, bigger males exhibit territorial-like behaviour, and those males, that are preferred
375 by females are more likely to release sperm closer to the eggs and hence the ovarian fluid

(Pyron 2003; Skinner and Watt 2007; Spence et al. 2007; Uusi-Heikkila et al. 2012). The differences in sperm longevity we found among males (indicated by the male effect in the main model, see table 1) may reflect a differential investment according to the male social environment. Earlier studies in the zebrafish showed that sperm traits vary with the intensity of male-male competition and male social status and affect the survival and performance of the resulting offspring (Zajitschek et al. 2014; Zajitschek et al. 2017), possibly due to the higher mutational load in sperm of stressed males (Silva et al. 2019). In addition, longer-lived sperm selected within ejaculates sire embryos with higher survival and adult fitness in zebrafish (Alavioon et al. 2019) and the ovarian fluid might be reinforcing selection for longer-lived sperm phenotypes, exacerbating the existing variation among males and possibly facilitating fertilization of the preferred/dominant male.

Despite increasing attention on ovarian fluid as a mediator of cryptic female choice, its proximate mechanisms remain elusive. Several of its components have been suggested to affect sperm performance such as egg-derived soluble factors, proteins, peptides, and RNAs, which may interact with proteins and peptides expressed on the sperm surface or in the seminal fluid (Kekäläinen and Evans 2018). Soluble factors released by eggs such as peptides secreted by egg jelly drive sperm chemoattraction in echinoderms (Darszon et al. 2004) and in several species of externally fertilizing fish (Yanagimachi et al. 1992). Similarly, in the mussel, *M. galloprovincialis*, differential attraction of sperm from the most compatible male is attributable to egg-derived chemical factors (Evans et al. 2012; Lymbery et al. 2017; Oliver and Evans 2014). In the zebrafish, follicles containing mRNA and serpin-type protease inhibitors have been found in the ovarian fluid (Knoll-Gellida et al. 2006; Minin and Ozerova 2015), and such inhibitors have been identified as mediators of post-mating mechanisms in

400 other species (Dosselli et al. 2018). In addition, the osmolality of the ovarian fluid is critical
401 for sperm functionality in the fertilization micro-environment and is comparable to that
402 found in salmonids (Wilson-Leedy et al. 2009). Interestingly, in the chinook salmon the ionic
403 composition and viscosity of the ovarian fluid have been found to vary among different
404 females with correlated effects on sperm motility and longevity (Rosengrave et al. 2009b).
405 Finally, variations in the rheological properties (i.e. viscosity) of the ovarian fluid might
406 contribute to explain the effects of ovarian fluid on sperm traits, selecting among sperm or
407 ejaculates exhibiting different motility traits (Lauga et al. 2007; Rosengrave et al. 2009b).
408 Future studies in this species will help shed light on both evolutionary patterns and
409 proximate mechanisms of the ovarian fluid effects in post-mating sexual selection. The
410 range of molecular tools developed for zebrafish will help reveal which molecular factors in
411 the ovarian fluid affect the sperm traits tested here.
412
413 In conclusion, our findings add robust data to the growing body of evidence for the role of
414 ovarian fluid in post-mating sexual selection as a means of cryptic female choice. We
415 showed that the ovarian fluid of individual females differentially affects sperm of individual
416 males. What we currently do not know is how important the effects of ovarian fluid are in
417 relation to processes of sperm competition. Competitive trials will answer this question and
418 provide insights into the fitness consequences of these effects. Importantly, our study adds
419 new aspects of sperm trait analysis that have not been investigated in previous studies. By
420 comparing sperm traits in water and ovarian fluid, we were able to show patterns of sperm
421 activity decline over time that cannot be captured by analyses including only one time point.
422 These patterns measured for several sperm swimming traits over time suggest possible
423 trade-offs among those traits and variation in the response of sperm to different media may

424 indicate adaptive condition-dependent responses of sperm (and male) to their different
425 roles in sperm competition. In particular, ovarian fluid may help to exacerbate differences in
426 sperm motility according to both quality of males, compatibility between partners, and the
427 distance where sperm are released compared to eggs.
428

FUNDING

This work was supported by a grant from the Australian Research Council (Discovery Project no. DP170100840).

ACKNOWLEDGEMENTS

We thank Craig Purchase for his precious help with the CASA plugin for sperm velocity assays. We are grateful to Ben Ezzy and Wendy Hopper for their help with fish maintenance at the Western Australian Zebrafish Experimental Research Centre (WAZERC, University of Western Australia).

DATA ACCESSIBILITY

Analyses reported in this article can be reproduced using the data provided by Poli, F (2018) 10.6084/m9.figshare.7770056.

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662 FIGURE LEGENDS

663

664 **Figure 1**

665 Schematic representation of the North Carolina II experiment used to test for interactions
666 between sperm and ovarian fluid (for clarity only one block is depicted). Ejaculates of two
667 males (MA and MB) were tested with the ovarian fluid (OF) of two females (FA and FB), with
668 two replicates for each male-female pair.

669

670 **Figure 2**

671 Effect of ovarian fluid (solid line) compared to freshwater (dashed line) on different sperm
672 traits measured every ten seconds for the first minute after sperm activation (means ± SE):
673 (A) sperm curvilinear velocity VCL, (B) sperm linearity LIN, (C) sperm beat-cross frequency
674 BFC, and (D) sperm motility, proportion of motile sperm.

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677 TABLES AND TABLE LEGENDS

678

Table 1. Estimates and significance levels for random factors from linear mixed effect models: male ID, female ID and their interaction are shown for all sperm traits. D.f. = 21. The interacting effect of male x female ID was significant for all traits considered. Male ID had a significant effect on sperm longevity, indicating that some males produced intrinsically longer-lived sperm than other males.

Sperm trait	**Male ID**		**Female ID**		**Male ID x Female ID**	
	χ^2	P	χ^2	P	χ^2	P
velocity	29.905	0.094	0.420	~1	41.475	**0.005**
linearity	5.809	0.999	5.523	0.999	39.264	**0.009**
motility	19.151	0.576	1.703	~1	34.193	**0.035**
beat cross frequency	24.245	0.281	1.890	~1	40.346	**0.007**
longevity	6.311	**0.012**	0.577	0.448	5.035	**0.025**

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