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REVIEW

Associations between Ovarian Fluid and Sperm Swimming Trajectories in Marine and Freshwater Teleosts: A Meta-Analysis

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ABSTRACT

Marine and freshwater spawning environments present fish sperm with unique challenges, but for both, gametes often signal prior to contact via biochemical interactions through maternally-derived compounds (i.e. eggs and ovarian fluid; OF). For example, when OF is incorporated into the fertilization environment, sperm have been observed to exhibit changes in swimming trajectories (e.g. motility and velocity), yet it remains unclear whether the presence of this OF consistently improves sperm performance. The objectives of this study were to determine the overall effect of OF on sperm performance using meta-analysis. Published literature was searched for studies comparing sperm motility and/or velocity in the presence and absence of OF. For each study, the log response ratios (lnRR) was calculated, in which positive values indicate improved performance in the presence of OF. For motility, the overall effect size was non-significant (lnRR = 0.09, CL = −0.06, 0.24), whereas velocity was positively affected by OF (lnRR = 0.10; CL = 0.04, 0.17). When segregated by environment, for freshwater species there was a significant positive effect of the OF on velocity (lnRR = 0.18, CL = 0.07, 0.29), which translated to an increase in velocity of 20%. In contrast, no effect was detected for velocity in marine species (lnRR = −0.01, CL = −0.02, 0.01). Overall, there is evidence that OF improves sperm performance, although spawning environment and/or taxonomic factors are likely to moderate these sperm-OF interactions. Together, these results further our understanding of natural reproductive processes governing sperm performance, mating systems, and fertilization dynamics.

KEYWORDS

fertilization dynamics; reproduction; cryptic female choice; gamete biology; sexual selection

Introduction

Fish are the largest group of living vertebrates with ~33,500 species (www.fishbase.org), all of which exhibit diverse reproductive strategies (Patzner 2008; Smith and Wootton 2016). Such diversity is fascinating for comparative biology in which many fish species have been appointed as biological models for various scientific disciplines. The spawning environment is often identified as a cause of variation in reproductive biology and specifically how gametes interact. During the last decade renewed attention has been paid to examine gamete interactions not only within a single species/group but also across different phylogenetic lineages. As a result of this effort, knowledge of gamete quality and fertilization dynamics for a variety of fish species (both cultured and wild) has increased, but many questions still remain to be resolved. It is fascinating to clarify the diversity among different gametes, their physiology, and what evolutionarily enabled them to adapt to environmental change. Sustainable management of fish populations, both *in situ* and within a production setting, requires an intimate working knowledge of gamete biology and the factors affecting fertilization (Andrews and Kaufman 1994; Mylonas et al. 2010). Domestication and commercial culture for many important fish species are impeded by difficulties with low fertilization. Therefore, increased knowledge of gamete biology is likely to make a significant contribution to species under investigation and for fish reproduction overall. Now being aware of the necessity to provide an

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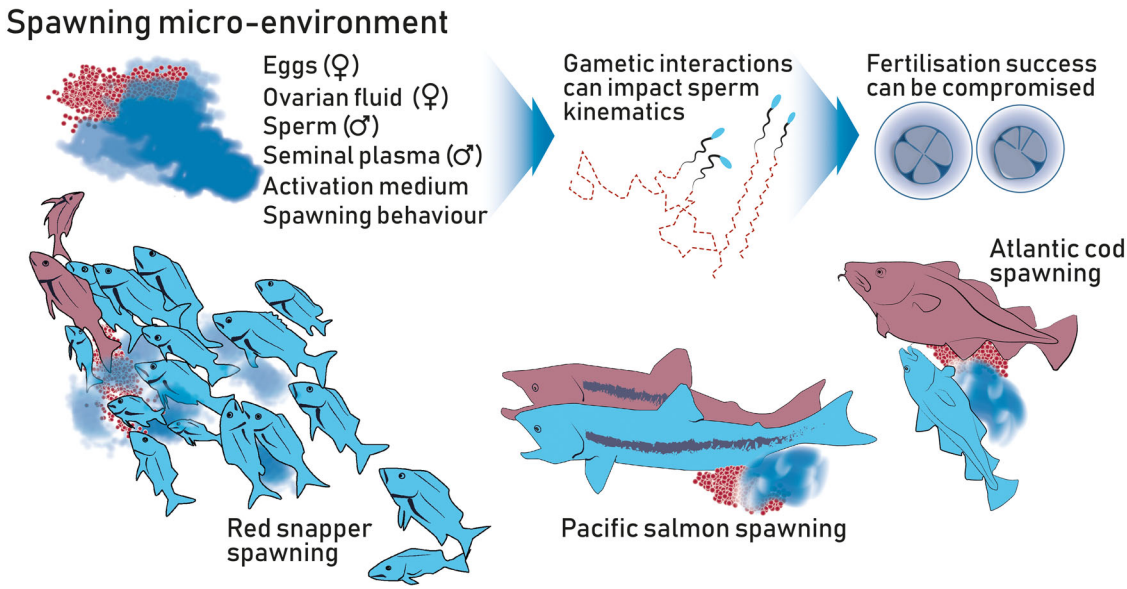

Figure 1. Representation of spawning dynamics for various fish species (e.g. Atlantic cod Gadus morhua, Pacific salmon Oncorhynchus spp., and Red snapper Lutjanus campechanus). In each scenario, females create unique fertilization micro-environments by expelling their distinct ovarian fluid (OF) along with an egg batch. In turn, these maternally-derived substances can potentially impact the outcome of a fertilization event by modifying sperm trajectories.

integrated view of sperm and oocyte communication mechanisms, the present study has been entirely devoted to shed light on the impact of maternal components (e.g. ovarian fluid (OF)) on sperm performance in two distinct groups of teleosts: marine and freshwater.

An overview of fertilization dynamics across fish taxa

Fish ontogeny initiates with fertilization, or more precisely, with egg activation. Generally, in teleosts, eggs and sperm develop separately within the male and female sexes. In some hermaphrodite species, however, individuals exhibit maturation of both parts of the ovotestis simultaneously, making them both male and female from a functional viewpoint (Coward et al. 2002). Fertilization in its broadest sense refers to the steps leading to and resulting in the fusion of the nuclei of the male and female gametes to form a diploid zygote (Kunz 2004). Many of the processes that happen during teleost fertilization seem similar to those seen in marine invertebrates and mammalian eggs. During the process of fertilization, sperm make essential contributions by transmitting paternal genetic material and initiating key intracellular signaling processes within the oocyte with consequences for egg activation and development (Whitaker and Swann 1993; Babin et al. 2007).

In teleosts, fertilization can occur either inside (internally) or outside (externally) of the female reproductive tract. In oviparous species eggs are ovulated from the ovarian follicles into the ovarian lumen or peritoneal cavity, usually following completion of the first meiotic division. In these species, once the mature egg has attained the metaphase of the second maturation (meiotic) division, it is released into the external aquatic environment with associated OF and then are subsequently fertilized (Figure 1). Afterward, embryonic development takes place outside of the maternal body cavity (Coward et al. 2002; Kunz 2004). External fertilization is by far the most common reproductive strategy in teleosts (Patzner 2008). In teleosts with internal fertilization (e.g. live-bearing fish such as swordtails and platyfish of the genus *Xiphophorus*), males transfer the sperm packets (usually by a modified anal fin) into spermatophores to the female receptaculum seminis of the genital tract. Afterward, the sperm may be retained for up to several months after insemination (Huang et al. 2004) and initiate fertilization in the female reproductive tract (Iwamatsu 2000). In these species, sperm are motile in OF and enter the ooplasm of intrafollicular oocytes in order to induce egg activation via the cortical reaction within the ovary (Iwamatsu 2000). Generally, internal fertilizers that give birth to live offspring are sub-classified as ovoviparous (eggs are incubated in a modified oviduct of the female) or viviparous (eggs develop in the ovary or the uterus after internal fertilization) (Coward et al. 2002).

Despite the reproduction strategy implemented, whether it be internal or external fertilization, eggs (particularly for freshwater species), can lose their fertilization ability very rapidly (Yamamoto 1961) due to

plugging of the micropyle (Kunz 2004), and thus, a sperm cell must enter quickly (Yanagimachi et al. 2017). Collectively, the capacity to fertilize varies across fish taxa and this variation has been attributed largely to genetic variability, intra-testicular aging of sperm, seasonality, breeding state, and differences in reproduction strategies (Scott and Baynes 1980; Coward et al. 2002).

Sperm activation, swimming trajectories, and fertilization success

In teleosts, sperm exhibits a remarkable variety of adaptations. Sperm cells are ejaculated into the external environment to spend their pre-fertilization lives fundamentally as free-living organisms, and they have adapted to specific fertilization environments depending on species (Pitnick et al. 2009). Generally, marine and freshwater environments each present sperm with unique challenges, causing differences in morphology, kinematic characteristics (i.e. motility, velocity, linearity), and biological processes (i.e. ATP usage). Differing activation mechanisms are also seen between these habitats (Suquet et al. 1994; Cosson 2004). In externally fertilizing fishes, it is common knowledge that sperm cells are immotile in the testes until activation is triggered upon release into the ambient environment (Morisawa et al. 1983), yet there is tremendous variation in how activation occurs. The major signals that may activate the motility of released or ejaculated sperm are changes in concentrations of inorganic (e.g. CO_2, H^+, K^+, Na^+, Ca^{2+}) and organic compounds. In addition, non-chemical factors such as osmolality and temperature are involved in triggering sperm motility (Alavi and Cosson 2005). For marine teleosts sperm become motile once they are released into hypertonic environments, but for freshwater species activation occurs adversely in hypotonic environments (Alavi and Cosson 2005; Alavi et al. 2007; Morisawa 2008). Interestingly, in some species such as medaka, *Oryzias latipes,* motility of sperm can be achieved by hypertonic, isotonic, or hypotonic solutions, depending on if the fish is acclimatized to a freshwater or seawater environment (Yang and Tiersch 2009). This phenomenon has also been observed in tilapia, *Oreochromis mossambicus,* which can reproduce in both freshwater and seawater. The sperm of freshwater-acclimated tilapia exhibits motility only in hypotonic conditions, but sperm of seawater-acclimated tilapia is motile in both hypertonic and hypotonic conditions (Morita et al. 2004). Typically, sperm from marine species have longer longevity than those of freshwater species likely due to differences in the physical properties of the activation media and reproductive strategies of each spawning type (Kime et al. 2001). Interestingly, species with internal fertilization exhibit much longer windows of motility. The viviparous guppy, for instance, displays longevity in seminal plasma for periods up to 48 h (Billard 1978). One common feature across species seems to be the importance of sperm swimming speed during each activity period; the average sperm velocity being 140 μm/s (ranging from 70 to 220 μm/s) for marine and 135 μm/s (ranging from 50 to 250 μm/s) for freshwater species (Browne et al. 2015).

Sperm quality in fishes can be assessed in many ways, but regardless of spawning type, sperm motility (percentage of motile sperm) and velocity are viewed as primary determinants of reproductive success. As such, these traits are commonly used to assess male gamete quality and fertilization potential (Gage et al. 2004; Rurangwa et al. 2004; Gallego and Asturiano 2018; 2019). Within the motile portion of an ejaculate, variation in sperm velocity could impact fertility potential of individual sperm cells (Pizzari and Parker 2009). Typically, sperm with higher velocity have the advantage of reaching the micropyle within a shorter window of time. Having a greater number of actively swimming cells per ejaculate also increases the chances of a single sperm achieving fertilization (Cosson et al. 2008). Increased sperm velocity and motility, thus, provide a crucial advantage for fish that spawn in highly competitive environments. Accordingly, in many externally fertilizing species, studies have reported positive correlations between sperm motility and fertilization success (Lahnsteiner et al. 1998; Casselman et al. 2006; Gallego et al. 2017), and similarly, reductions in sperm motility result in decreased reproductive success (Burness et al. 2004; Casselman et al. 2006). Like motility, higher rates of fertilization and paternity were also achieved with higher sperm velocities for both marine and freshwater species, such as Atlantic salmon, *Salmo salar* (Gage et al. 2004), bluegill, *Lepomis macrochirus* (Burness et al. 2004), Atlantic cod, *Gadus morhua* (Rudolfsen et al. 2008), and green swordtail, *Xiphophorus helleri* (Gasparini et al. 2010). For internally fertilizing species, prolonged sperm is retained in the female reproductive tract, and sperm migration toward the ovum may be influenced more by the female reproductive tract than sperm swimming behavior directly (Pizzari and Parker 2009).

How do sperm and oocytes communicate before fertilization events?

Fish have evolved a diversity of strategies and a wide range of processes that lead up to fertilization to

maximize their chances of reproductive success (Coward et al. 2002; Smith and Wootton 2016). Fertilization depends on successful interactions between eggs and sperm during spawning. As such, there has always been keen interest in gamete interactions, specifically how the oocyte, sperm, and maternally-derived substances from the female reproductive tract cooperatively impact fertilization success. The specific mechanisms underlying these interactions remain elusive for most species. It seems this complex chemical dialogue between eggs and sperm provides the scope for female-induced sperm recognition and selection, ensuring that only a small subset of sperm cells have the opportunity to reach the fertilization site (Fitzpatrick and Lüpold 2014). Sperm are usually produced and released more abundantly than eggs (e.g. billions of spermatozoa during spawning) yet sperm-egg fusion occurs at a ratio of 1:1. Therefore, sperm are always subject to strong selection or competition (Parker and Pizzari 2010; Parker and Lehtonen 2014; Matsuzaki et al. 2018). In order to fully understand how competition occurs, we must first answer a question: How do sperm find the egg? Sperm ejaculated into either the female genital tract or external environment do not likely reach the oocyte by coincidence, and the fact that very few succeed in making their way to the oocyte points to sperm guidance factors. Once sperm cells are activated, they must compete to be the first to locate an oocyte and find the entry site on the egg plasma membrane (Yanagimachi et al. 2013; Browne et al. 2015). In order to do this, they must first interact with either the female's reproductive tract or maternal components in the vicinity of the micropyle such as OF. This fluid acts as a guide by stimulating sperm to undergo numerous physiological, biochemical, morphological, structural, and behavioral modifications (Oda et al. 1995; Coward et al. 2002; Yanagimachi et al. 2013). Maternal components acting as sperm attractants have been studied in various fish species but also in mammals and invertebrates (Iwamatsu 2000; Yoshida and Yoshida 2011; Yanagimachi et al. 2013; Browne et al. 2015; Cosson 2015; Yanagimachi et al. 2017; Yoshida and Yoshida 2018). Accumulating evidence from different taxa show that both the female reproductive tract and oocyte secrete many factors such as sperm-activating peptides (e.g. polypeptides and glycoprotein), amino acids, small molecules, yolk lipids such as polyunsaturated fatty acid (PUFA), and nitric oxide which can act as chemo-attractants that "direct" sperm into the micropylar canal (Creech et al. 1998; Kubagawa et al. 2006; Cherr et al. 2008; Han et al. 2010; Yanagimachi et al. 2013, 2017). These substances are also known to aid in sperm chemotaxis, as initially described by Dan (1950) in a hydrozoan, *Spirocodon saltatrix*. Specifically, the matrix and the jelly coat encompassing the egg envelope (i.e. chorion) varies in different species and generally displays a prominent role in sperm-egg detection (Iwamatsu 2000). The swarming of numerous sperm cells in or near the micropyle during fertilization gives evidence that the egg chorion may release substance(s) (e.g. sperm motility-initiating factors (SMIFs), herring sperm activating proteins (HSAPs), PUFAs) that attract homologous sperm toward the egg (Hart 1990; Oda et al. 1998; Vines et al. 2002, Kubagawa et al. 2006; Cherr et al. 2008). Those sperm cells moving along the surface of the egg chorion have a higher chance of entering the micropylar canal than those swimming freely and randomly in the aqueous environment (Yanagimachi et al. 2013).

Collectively, changes in the swimming patterns of teleost sperm toward an egg may have something in common with the "chemotactic" movement of invertebrate sperm to reach an egg as well as hyperactivation of mammal sperm before fertilization. Sperm-oocyte communication in fish is quite diverse compared with other phyla, largely due to the diverse environments where gamete encounters take place, whether it be inside the female reproductive tract or immersed in huge volumes of maternal components mixed with water. Furthermore, the variability of gamete interactions across fish groups may also partly be due to different motility and morphology traits required for each fertilization environment and for penetration of the chorion (Browne et al. 2015).

Does ovarian fluid impact sperm swimming behavior and fertility?

Ovarian fluid, the substance containing maternal compounds, is secreted by female ovarian epithelial cells before or at the start of ovulation (Aegerter and Jalabert 2004). For the majority of external fertilizers, eggs are ovulated into ovarian cavities and spawned with an abundance of OF (the quantity of OF is variable across species) out of the genital pore through the short oviduct together with the cumulus-oocyte complex (Iwamatsu 2000). Previous models of gamete biology and sperm performance have largely focused on aqueous solutions that lack OF. As such, these studies do not necessarily represent the broader chemical environment in which sperm is likely to have evolved and may also overlook the effects caused by

each female's unique micro-environment. Sperm motility and velocity can be directly affected by physical properties of the activation environment (Kime and Tveiten 2002; Alavi and Cosson 2005; Cosson et al. 2008; Browne et al. 2015). Experimental protocols have been employed over the years to assess the importance of sperm-OF interactions (reviewed by Zadmajid et al. 2019). Results have shown that when maternally-derived compounds are incorporated in the fertilization environment, sperm exhibited increased motility and velocity in several fish species (Yoshida and Nomura 1972; Turner and Montgomerie 2002; Rosengrave et al. 2009a; Butts et al. 2012; Galvano et al. 2013; Geßner et al., 2017). For example, in the brown trout, *Salmo trutta fario*, incorporating OF into the activation environment enhanced sperm longevity to over 5 min and improved the fertilization success (Lahnsteiner 2002). For several other salmonids, sperm velocity was also elevated when cells were activated in OF as compared to water (Gage et al. 2004; Butts et al. 2012; Rosengrave et al. 2016). Furthermore, for ocean pout, *Macrozoarces americanus* (Yao and Crim 1995) and spotted wolffish, *Anarhichas minor* (Kime and Tveiten 2002), sperm remained motile for 24 to 48 h in OF and became immotile on contact with seawater. An interesting example of the influence of OF on sperm swimming behavior has been reported by Elofsson et al. (2003b) with the three-spined stickleback, *Gasterosteus aculeatus*: a teleost found inhabiting fresh, brackish, and marine waters. In this species, OF prolonged sperm longevity from both fresh and brackish water for up to 7 and 10 h, respectively, with some sperm found to be active for up to 24 h. Furthermore, in some species, OF enhanced sperm fertility more than in other activation media. For instance, for certain salmonids (e.g. lake trout, *Salmo trutta* and Atlantic salmon, *Salmo salar*) when sperm was diluted in water they had completely lost their fertilizing capacity at 40 s post-activation, while those in Ringer's solution showed 15.4% fertility after 2 min. Surprisingly, those diluted in OF fertilized 78.8% of egg after 5 minutes (Ginsburg 1963). Additionally, some studies have showed that OF had a limited or negative impact on sperm performance (Wojtczak et al. 2007; İnanan and Öğretmen 2015; Kleppe et al. 2018).

Although the mechanism by which OF enhances and/or inhibits sperm performance is not fully understood, OF encompasses an array of physical (i.e. color, volume, viscosity, pH, and osmolality), biochemical (e.g. Na^+, Cl^-, K^+, Mg^{2+}, Ca^{2+}, proteins) and organic constituents (metabolites and enzymes) that are known to influence sperm swimming trajectories (Turner and Montgomerie 2002; Woolsey et al. 2006; Wojtczak et al. 2007; İnanan and Öğretmen 2015; Alonzo et al. 2016). Components of the OF can also potentially influence ATP metabolism, thus increasing sperm longevity and velocity (Turner and Montgomerie 2002). In mammals, OF called follicular fluid has been proposed to induce chemotaxis in sperm thereby, changing sperm swimming behavior in several mammals such as humans (Ralt et al. 1991), mouse (Oliveira et al. 1999), rabbit (Fabro et al. 2002), and bovine (Gil et al. 2008).

Collectively, this complex chemical dialogue between OF and sperm provides the scope for sperm recognition by the female (Fitzpatrick and Lüpold 2014). This process offers a mechanism for sexual selection *via* cryptic female choice (CFC), which is defined as the ability of females to select specific genotypes/phenotypes or sperm characteristics of the male (Thornhill 1983). Sexual selection potentially alters the dynamics of sperm competition (Gasparini and Pilastro 2011; Butts et al. 2012; Makiguchi et al. 2016; Lehnert et al. 2017). For example, the ejaculates of various males may co-occur around a set of ova at the time of gamete interaction, resulting in intense sperm competition and selection (Parker 1970, 1984, 1998; Pizzari and Parker 2009). Such competition among individual males is now recognized as one of the most powerful evolutionary forces influencing reproductive outcomes for most taxa (Parker 1970; Birkhead and Pizzari 2002; Pizzari and Parker 2009; Kelly and Jennions 2011; Firman et al. 2017).

Motivation for meta-analysis on sperm-ovarian fluid interactions

Generalities about the magnitude and relevance of OF-sperm interactions across fish species are currently difficult to make. One dilemma lies in the variation of observed results among studies, which have made it difficult to draw broad conclusions across species. In such cases, meta-analysis provides a quantitative approach to systematically assess the degree and causes of heterogeneity among the results of individual studies and to derive an estimate of the magnitude of any overall biological effect (Huque1988; Haidich 2010). Therefore, the objectives of this analysis were to determine whether OF has an overall effect on sperm performance traits across fish species that

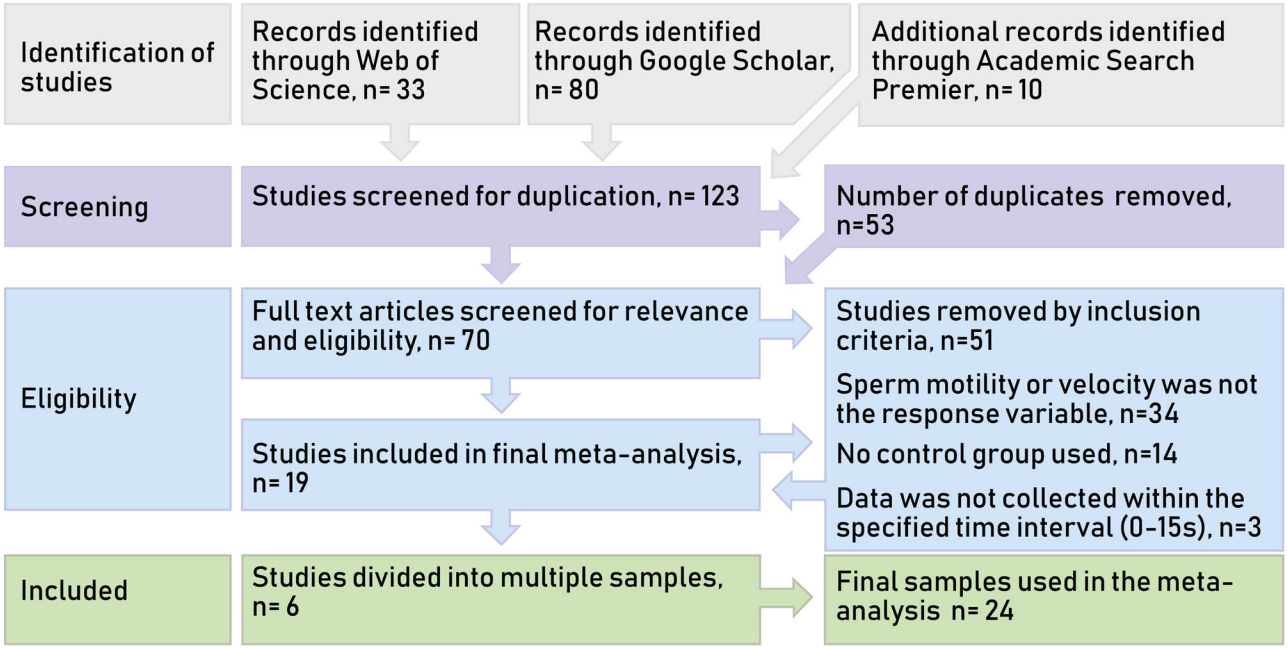

Figure 2. PRISMA flow diagram of studies for this meta-analysis on the effects of ovarian fluid on sperm performance. The chart describes the initial identification of studies across multiple databases, screening and elimination of duplicate findings, and eligibility criteria, resulting in a final inclusion of 19 separate articles divided into 24 study samples. Number of studies is indicated by "n" in each box.

spawn in freshwater and marine environments. This was done by systematically analyzing the current published studies on the effects of the OF on sperm motility (%) and velocity (μm/s). For each study, the log response ratio (lnRR) was calculated between control groups (without OF) and treatment groups (with OF). These effect size statistics were collated using meta-analytic models to determine the direction and strength of any overall relationships and to discuss potential causes for heterogeneity among the results reported by all studies.

Methods

Literature search and data extraction

Studies were collected systematically following PRISMA's best-practice protocols in three databases: Web of Science, Google Scholar, and Academic Search Premier. Research articles were searched from inception until 10 August 2018. The responses of OF were collected from studies using primary determinants of male reproductive success in fishes: motility and velocity (Lahnsteiner et al. 1998; Gage et al. 2004; Linhart et al. 2005). Keywords used were "ovarian fluid", "sperm", "sperm and/or motility", "sperm and/or velocity", "sperm and/or competition", "sperm and/or performance", and "cryptic female choice." To be included, studies must:

i. Be performed on a fish species.
ii. Report data on sperm motility and/or velocity; acceptable measures of sperm velocity included average path velocity (VAP), curvilinear velocity (VCL), and straight-line velocity (VSL), with the majority of studies (80%) reporting VCL. Due to collinearity between the different measures of velocity, only one measure of velocity was selected when more than one was reported in a study. 40% of studies reported more than one measure of velocity, but each of these studies reported VCL, therefore it was selected as the primary measure of velocity when available. As for the studies that did not report VCL, VAP was chosen as the primary measure. These measurements were analyzed collectively with VCL as a single unit of velocity (all expressed in μm/s) for this analysis.
iii. Include treatment groups, wherein one group or more consisted of activated sperm in the presence of OF and an equivalent control group of sperm was activated in a media designed to reflect the natural environment without OF (i.e. a full control group).
iv. Analyze motility and velocity within the first 0-15 s post-activation, which is the most critical window for fertility in fishes (Iwamatsu et al. 1991; Fleming 1996; Hoysak and Liley 2001;

Table 1. Final published studies (n = 19) that met our final inclusion criteria and were included for meta-analysis.

Publication	Species	Spawning habitat	OF (%)	Data reported
Beirão et al. (2014)	*Gadus morhua*	Seawater	5, 25	Motility, velocity
Beirão et al. (2015)	*Gadus morhua*	Seawater	5, 25	Motility, velocity
Butts et al. (2012)	*Salvelinus namaycush*	freshwater	20	Motility, velocity
Butts et al. (2017)	*Oncorhynchus tshawytscha*	freshwater	40	Velocity
Dietrich et al. (2008)	*Oncorhynchus mykiss*	freshwater	100	Motility
Diogo et al. (2010)	*Solea senegalensis*	Seawater	25, 50	Velocity
Elofsson et al. (2003b)	*Spinachia spinachia*	Seawater	25	Motility, velocity
Elofsson et al. (2003a)	*Gasterosteus aculeatus*	Seawater	25	Motility, velocity
Elofsson et al. (2003a)	*Gasterosteus aculeatus*	freshwater	25	Motility, velocity
Galvano et al. (2013)	*Salvelinus namaycush*	freshwater	10, 15	Motility, velocity
Gasparini and Pilastro et al. (2011)	*Poecilia reticulata*	freshwater	60	Velocity
Hatef et al. (2009)	*Salmo trutta*	freshwater	100	Motility
Inanan and Öğretmen et al. (2015)	*Oncorhynchus mykiss*	freshwater	100	Motility
Lahnsteiner (2002)	*Salmo trutta*	freshwater	100	Motility, velocity
Lehnert et al. (2017)	*Oncorhynchus tshawytscha*	freshwater	50	Velocity
Makiguchi et al. (2016)	*Oncorhynchus masou*	freshwater	10	Motility, velocity
Rosengrave et al. (2009b)	*Oncorhynchus tshawytscha*	freshwater	50	Motility, velocity
Turner and Montgomerie et al. (2002)	*Salvelinus alpinus*	freshwater	50	Velocity
Yeates et al. (2013)	*Salmo salar, Salmo trutta*	freshwater	100	Motility, velocity

Studies either reported sperm motility, velocity, or both as response variables. Additional information on spawning habitat and % ovarian fluid (OF) used was collected to assess their respective effects on the RMA. Studies indicated by a and b are separate studies within the same year, and duplicate letters represent multiple samples within the same study (one for freshwater and one for marine). Studies with multiple species or OF concentrations listed were also divided into independent samples for the analysis.

Yeates et al. 2007). Data was collected only within this interval to investigate the impacts during the most influential period of sperm activation and fertility.

v. Report all of the following (or data from which they can be derived) for all treatment groups; means, standard deviations (SDs), and sample sizes.

After first screening by title and abstracts and then by full text for the aforementioned criteria, there were 19 studies included in the meta-analyses (Figure 2; Table 1). From each study, data was collected on means, SDs, and sample sizes in treatment and control groups from tables, text, and figures. The studies gathered used subtly different designs, where replication was designed to capture/control different sources of variation. For example, some studies pool the OF from several females and compare the performance of sperm from individual males in presence/absence of this fluid, meaning replicates capture among-male variation. In other cases, the sperm of males was pooled and tested against the fluid of individual females, thus capturing among-female variation. Nevertheless, in all cases the relative effect of OF on mean sperm function in each group is comparable among all studies.

Where data were reported in figures, they were extracted manually using WebPlotDigitizer v.3.9 (WebPlotDigitizer, Austin, TX, USA). When multiple data points were reported within the critical 0-15 s window, all data points were extracted and subsequently collated as described below. For any missing data, attempts were made to contact the authors, and thereafter, their inputs were included. Data on moderator variables (study-specific factors) was also collected, which might be expected to influence the sign or magnitude of the OF effects on sperm traits. Those variables were (*i*) taxonomic data on the study species (families), (*ii*) whether fish spawned in fresh or saltwater environments, and (*iii*) OF concentration.

Effect size calculations

For meta-analysis, the results of different studies must be combined via effect sizes that are on a common scale, which summarize the sign and magnitude of the effect reported in each study. Where studies report data on traits measured in different units (e.g. measures of sperm velocity) a standardized, unitless effect size is required. Here, the log response ratio (lnRR) was implemented, which is the natural logarithm ratio of the means in the two groups. The lnRR (and its associated sampling variance; s^2_{lnRR}) were calculated using the "escalc" function, in the package *metafor* (Viechtbauer 2010) in R Studio v.1.1.383 (RStudio, Boston, MA, USA). R Studio was used for all analyses unless otherwise stated. For ecological/evolutionary meta-analyses such as this, the lnRR and also the standardized mean difference (SMD) are both commonly used (Nakagawa and Santos 2012; Senior et al. 2016a). The lnRR was used rather than the SMD as the latter is standardized in units of pooled standard deviation (SD). As discussed above, the studies synthesized here have slightly different designs which capture different sources of variation, rendering the

SDs of different studies incomparable (although the means remain comparable). Unlike the SMD, the interpretation of effect magnitude for lnRR is not affected by the SD of the study. Effect sizes were calculated such that positive values indicate that the measure of interest is greater in the presence of OF. To aid interpretation, in places the overall estimates of the lnRR were back transformed, yielding the estimated difference ratio of the means between control and treatment groups.

For studies where data were reported over multiple time points, all means and SDs reported within the 0-15 s window were combined into a single measurement following Higgins and Green (2011). In cases where there were more than one treatment group compared to the same control group (such as OF from two different fish populations), the sample size of the control group was divided by the total number of treatment groups to ensure the n in the control group was not over-represented.

Elofsson et al. (2003b) conducted trials on freshwater and marine sticklebacks within the same species (*Gasterosteus aculeatus*). Additionally, Yeates et al. (2013) conducted independent experiments within the same study on two different fish species: trout, *Salmo trutta,* and Atlantic salmon. As such, data points for each species were treated as separate samples for both studies. It is to note that the study also employed the use of conspecific OF (within the same species) as well as heterospecific OF (from different species) as an independent variable. For these analyses, only data for conspecific OF was assessed to maintain consistency with other studies. Additionally, within study divisions were made, such that studies analyzing >1 OF concentration was treated separately due to their potential to cause variations in effect size and to minimize within-study variation. In such cases, again, the n of the control group was still divided by the number of treatment groups before pooling the data, as stated previously. Sperm has been shown to behave differently depending on whether OF is present in high or low concentrations in the activation environment (Diogo et al. 2010; Beirão et al. 2014). Therefore, after separation by OF concentration, data points were then processed using the method described above. In total there remained 24 pairwise effect sizes derived from 19 published studies.

Statistical analysis

Effect sizes were analyzed using random-effects meta-analytic models (REMAs), which are used to: 1) estimate the overall sign, magnitude, and statistical significance of effects, and 2) estimate the degree of heterogeneity among the effects. REMAs were considered more applicable than fixed-effect meta-analysis (FEMA), as REMAs do not assume that all effect sizes are drawn from the same statistical population (Nakagawa et al. 2017) and therefore allow for heterogeneity among the effect sizes reported by different studies. In the current case, heterogeneity is likely to be present as we have data drawn from several different species (Senior et al. 2016a). REMAs were implemented using the 'rma' function in *metafor* and estimated *via* restricted maximum-likelihood estimate (REML) (Viechtbauer 2010). Outcomes were measures of lnRR, and sampling variance was specified as s^2_{lnRR}. REMA (as opposed to fixed-effects meta-analysis) and related multi-level meta-analysis are generally the most applicable methods because they allow for heterogeneity, which is expected in almost all biological datasets (Gurevitch and Hedges 1999). Heterogeneity may be attributable to many sources, but in studies in which each effect size comes from a different species or biological system, inter-specific differences are expected to impact the results (Senior et al. 2016a). Separate REMAs were implemented for lnRR of motility and velocity. Overall estimates of effect with 95% confidence limits (CL) not spanning zero were considered statistically significant. Heterogeneity was quantified using the Q test (Cochran 1954), which provides a test for the presence of statistically significant (i.e. non-zero when $p < 0.05$) heterogeneity. I^2 values were also reported, which measure the percentage of variance among effect sizes due to true heterogeneity (Huedo-Medina et al. 2006) (i.e. not due to sampling variance). Values of I^2 are somewhat arbitrarily defined, but 25, 50 and 75%, are typically taken to represent low, medium, and high heterogeneity, respectively (Higgins and Thompson 2002), although in a multi-species study such as ours estimates of around 85-90% are common (Senior et al. 2016a).

An additional source of heterogeneity and non-independence in multi-species analyses such as this is phylogenetic covariance, wherein more closely related species may be expected to more closely represent one another owing to a shared evolutionary history (Harvey and Pagel 1991; Pagel 1999; Nakagawa and Santos 2012). The effects of such shared evolutionary history can be quantified as $I^2_{\mathrm{Phylogeny}}$ (the percentage of among-effect size variation due to shared evolutionary history; similar to Pagel's λ; (Pagel 1999)) using phylogenetic multi-level meta-analysis

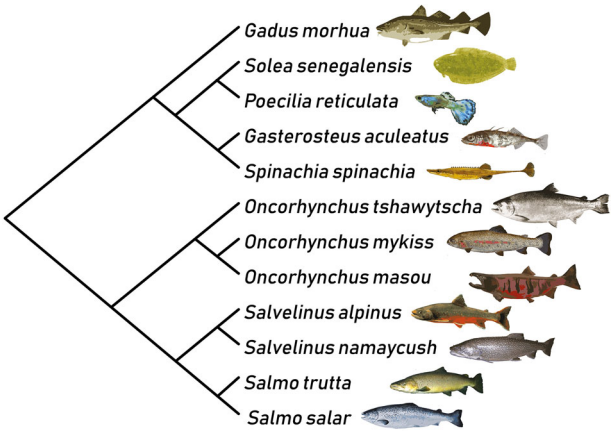

Figure 3. Genetic relatedness phylogeny diagram for the fish species included in the meta-analysis using the interactive Tree of Life-based on the National Center for Biotechnology Information (NCBI) taxonomy. Note that many species shared closely related phylogenies (consisted primarily of salmonids).

(PMLMA; (Nakagawa and Santos 2012; Hadfield and Nakagawa 2010)). Such PMLMAs are an extension of REMAs, which allow for multiple sources of heterogeneity (such as correlated evolutionary history) to be modeled as random-effects in a very similar manner to generalized linear-mixed models, as are commonly used in the analysis of primary data (Nakagawa and Santos 2012; Hadfield and Nakagawa 2010). A phylogeny was created for the species included in the analysis using the interactive Tree of Life (iTOL; http://itol.em … bl/de/), generating a tree based on data from the National Center for Biotechnology Information (NCBI) taxonomy (Figure 3). The phylogeny was converted to an ultra-metric format following Grafen's method (Grafen 1989) with $rho = 1$, and associated covariance matrix for effect sizes under a Brownian motion model of evolution was included in the PMLMA as a random effect using rma.mv function. From the estimated variance components $I^2_{\text{Phylogeny}}$ was calculated following Nakagawa and Santos (2012).

To explore whether the recorded moderator variables (see *Literature Search and Data Extraction* above) explained heterogeneity, first, separate REMAs were fitted for each spawning type (freshwater vs marine) to get independent overall effect sizes for each. Differences were then tested between spawning types using random-effects meta-regression (REMR), again implemented using the 'rma' function, as well as for linear effects of OF concentration. Effects of moderators were determined to be significant when based on the Q-test for moderators ($p < 0.05$).

Publication bias occurs when processes of publication systematically cause the under/over-representation of studies with specific outcomes relative to the total pool of studies performed. This creates meta-analyses based on the published literature but generates biased estimates (Møller and Jennions 2001). A common occurrence is when there is a tendency for studies with significant and/or large effects to have a higher rate of submission and acceptance (Egger et al. 1997; Rothstein et al. 2006). A common method of testing for publication bias is creating a funnel plot that displays the magnitude and direction of each effect size collectively. Publication bias assumes that if most of the studies are clustered on the positive side (giving it an asymmetric appearance), there are assumed to be negative effect sizes that were not included in the analysis which may potentially skew the overall effect size of interest. In addition to visually analyzing funnel plot symmetry, here a trim-and-fill analysis was utilized, as described by Duval and Tweedie (2000). This statistical method generates each REMA as before but estimates the number of potentially 'missing' effects and any potential bias in the overall estimated effect caused by the exclusion of those studies. Trim and fill analyses were implemented using the 'trimfill' function in *metafor*.

Results

Motility

Data for motility was collected from 14 different studies, yielding a total of 18 effect sizes (Figure 4). Of these, 12 were for freshwater and 6 for marine species. The results of the REMA for the full analysis gave an overall effect size of lnRR = 0.09 (CL = −0.06, 0.24), which did not deviate significantly from 0. There was high heterogeneity between studies ($Q = 301$, df = 17, $p < 0.001$; $I^2 = 97.7\%$), matching previous meta-analytic results for sperm motility in which $I^2 = 92.5\%$ (Senior et al. 2016b). The PMLMA estimated that total heterogeneity was not attributed to shared evolutionary history ($I^2_{\text{Phylogeny}} < 0.01$).

When analyzed individually by spawning environment, freshwater (12 effect sizes) and marine (6 effect sizes) had overall lnRR = 0.13 (CL = −0.09, 0.36) and lnRR = −0.02 (CL = −0.06, 0.02), respectively ($I^2_{\text{Fresh}} = 98.5\%$, $I^2_{\text{Marine}} = 0\%$), perhaps indicating sperm for freshwater species exhibited a greater positive response to OF than for marine species. Nevertheless, a direct comparison using REMR detected no statistically significant difference between groups ($Q_{\text{Moderator}} = 0.26$, df = 1, $p = 0.61$). REMR

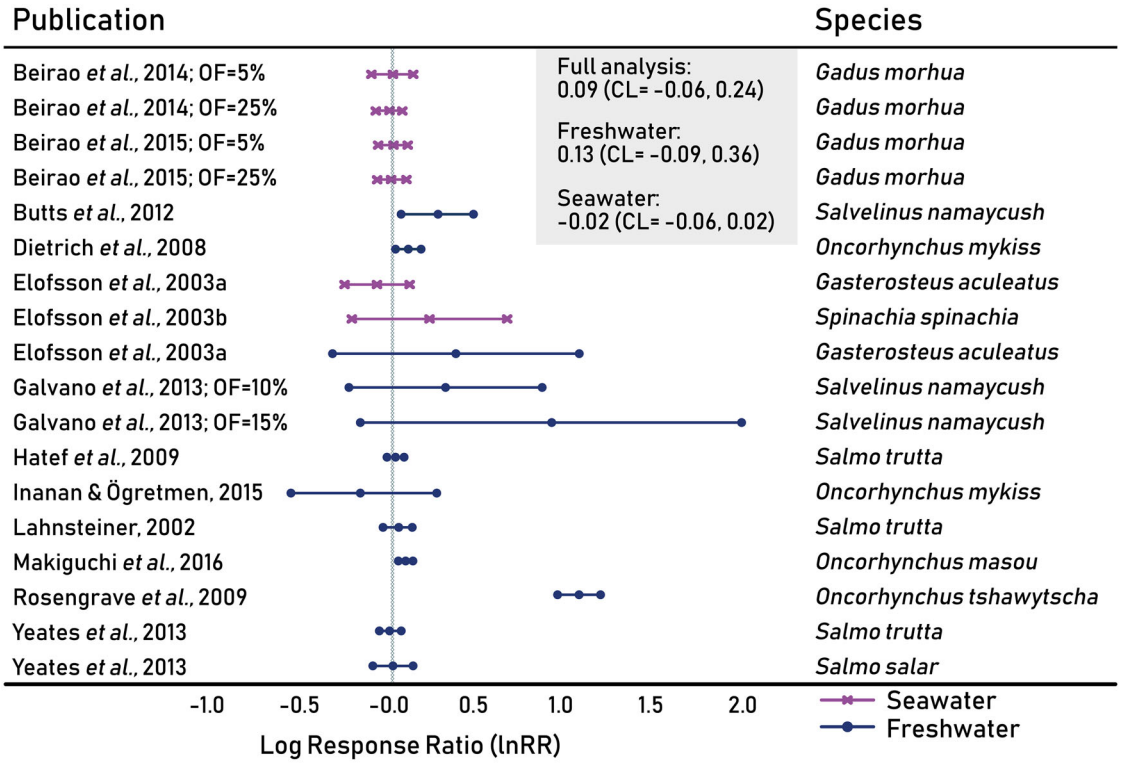

Figure 4. Forest plot for the effects of ovarian fluid (OF) on sperm motility (n = 18 effect sizes). Study ID is given on the left and species of fish used in each is listed on the right. Effect sizes are given as log response ratios (lnRR) with 95% confidence limits for all species. Effect sizes with confidence intervals that do encompass 0 are not statistically significant.

for OF concentration also had no significant effect on effect magnitude ($Q_{\text{Moderator}} = 0.84$, df = 1, p = 0.36). The estimated slope of the relationship was effectively zero (slope for 1% increase in OF = −0.002, CL = −0.006, 0.002).

Publication bias was inspected visually by analyzing asymmetry in the funnel plot (Figure 5A). Most of the studies reported either had no effect or small-sized effects in the positive direction, but there were some negative effects (primarily from the marine studies) that balanced the distribution to the left. There were 3 outliers outside the confidence interval region, but neither of them had reasons to be excluded from the analysis. The trim and fill method, however, did not estimate any studies to be missing from the left side, and results of the REMR remained unchanged with this modification.

Velocity

Data for velocity was collected from 16 different studies, yielding a total of 20 effect sizes (Figure 6). Of these, 12 were for freshwater and 8 for marine species. Across all studies, velocity was positively affected by OF. For the full analysis REMA, the lnRR was 0.10 (0.04, 0.17), which when back transformed equates to a ratio of 1.11, suggesting velocity is increased by 11% in the presence of OF. Again, however, heterogeneity was high ($Q = 100$, df = 19, $p < 0.001$; $I^2 = 91.0\%$) between studies. The PMLMA detected a low degree of phylogenetic heterogeneity ($I^2_{\text{Phylogeny}} = 31.4\%$), suggesting a mild correlation in effect among closely related species.

For freshwater species there was a statistically significant positive effect of OF (lnRR = 0.18, CL = 0.07, 0.29). Back transforming the overall estimate indicates a mean ratio of 1.20, suggesting OF increases velocity by 20% in freshwater species. The effect sizes within freshwater species were, however, heterogeneous ($I^2_{\text{Fresh}} = 86.7\%$). In contrast, we detected no effect of OF on velocity in marine species, and there was no heterogeneity among effect sizes (lnRR = −0.01, CL = −0.02, 0.01, $I^2_{\text{Marine}} = 0\%$). A direct comparison among groups using REMR detected a statistically significant difference in the effect of OF on velocity between marine and freshwater species ($Q_{\text{Moderator}} = 7.7$, df = 1, $p < 0.01$). The REMR of OF concentration did not detect a significant impact on effect sizes across studies ($Q_{\text{Moderator}} = 1$, df = 1, p = 0.32) and had a negligible slope (slope for 1% increase in OF = 0.002, CL = −0.001, 0.004).

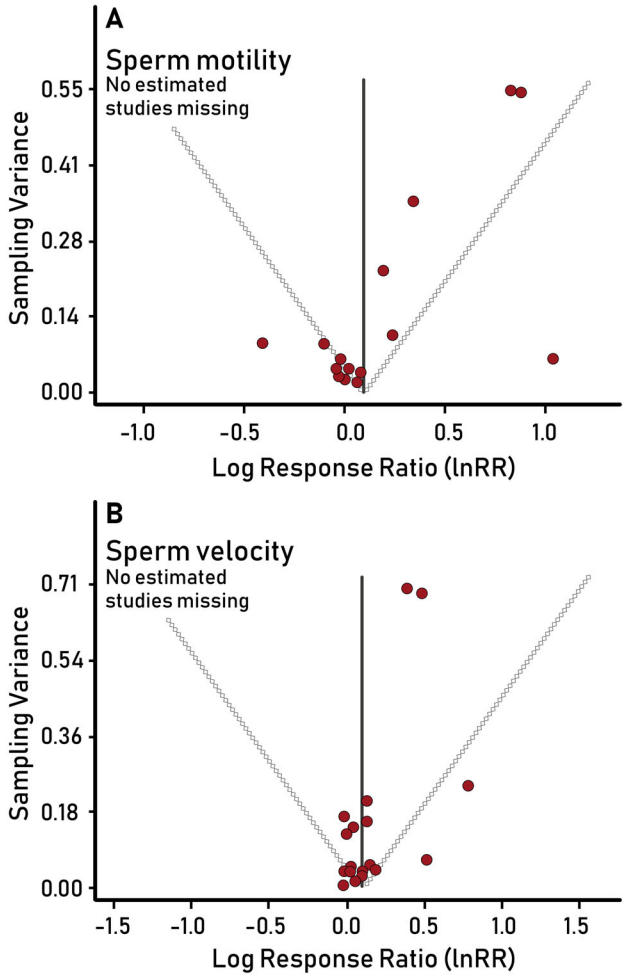

Figure 5. Funnel plots constructed to verify the presence of publication bias for each trait for (A) sperm motility and (B) sperm velocity. Dashed lines represent meta-analytic mean of the lnRR as estimated by the RMA analysis. Due to funnel asymmetry around the means, the number of studies estimated to be missing from a given side was assessed with the 'trimfill' function.

Publication bias was not detected for velocity (Figure 5B). The shape of the funnel plot suggested that there are two outlying large positive effects beyond the confidence interval region. When the trim and fill function was applied, there were no studies estimated to be missing from the left side, and the results were unchanged with this modification. Thus, it can be concluded that the results were not overestimated or misinterpreted due to publication bias.

Discussion

The receptivity of teleost gametes is rather short, thus fertilization through the egg micropylar canal must occur rapidly (Yanagimachi et al. 2017). Upon sperm contact with activation media, the cells become vigorously motile until cessation and have been shown to be greatly influenced by maternally-secreted compounds. Results from this meta-analysis show that the presence of OF in the fertilization environment has an impact on sperm swimming trajectories and that this impact varies between spawning types (i.e. freshwater or marine). More specifically, for freshwater species there was an overall positive effect of the OF on sperm velocity, while no effect was detected for the marine species.

Generally, all but a few studies reported positive effects for sperm velocity. For that reason, studies that reported negative effect sizes are further considered here in detail. Beirão et al. (2014,2015) concluded that OF inhibited sperm performance in Atlantic cod, contradictory to the findings of Litvak and Trippel (1998) on the same species. Litvak and Trippel (1998) reported increased sperm motility and swimming speeds in OF. However, Beirão et al. (2014, 2015) reported that the control (seawater without OF) yielded better sperm performance despite female origin (captive vs. wild or native vs. foreign population) or OF concentration used (5 or 25%). The authors attributed their findings to differences in methodology, such as using previously frozen OF samples instead of freshly collected samples as in the prior study, which could have altered some of its physical properties. There could have also been changes in osmolality and pH of the OF between experiments and potential mechanisms of cryptic female choice. Whether the results are due to one or all these factors remains unclear. In this example and in all studies considered, it is important to be cognizant of all sperm activating factors (i.e. temperature, pH, osmolality; reviewed by Alavi et al. 2008) that may impact the results.

Heterogeneity is almost always expected in ecological studies of this sort, so a number of effects were statistically tested as moderators using meta-regression. This approach is commonly used to explain sources of the high heterogeneity in meta-analyses and confirms the robustness of the results (Nakagawa and Santos 2012). Potential impacts of spawning type were explored, OF concentration, and shared evolutionary history (Hadfield and Nakagawa 2010). Differences in life-history patterns, evolutionary aspects, and reproductive strategies across species within considerably different evolutionary lineages introduced the potential for heterogeneity within our analysis. In this case, groups comprised of popular, hatchery-reared species of economic value; for freshwater species in particular. This resulted in most of the data originating from salmonids. Evidence for

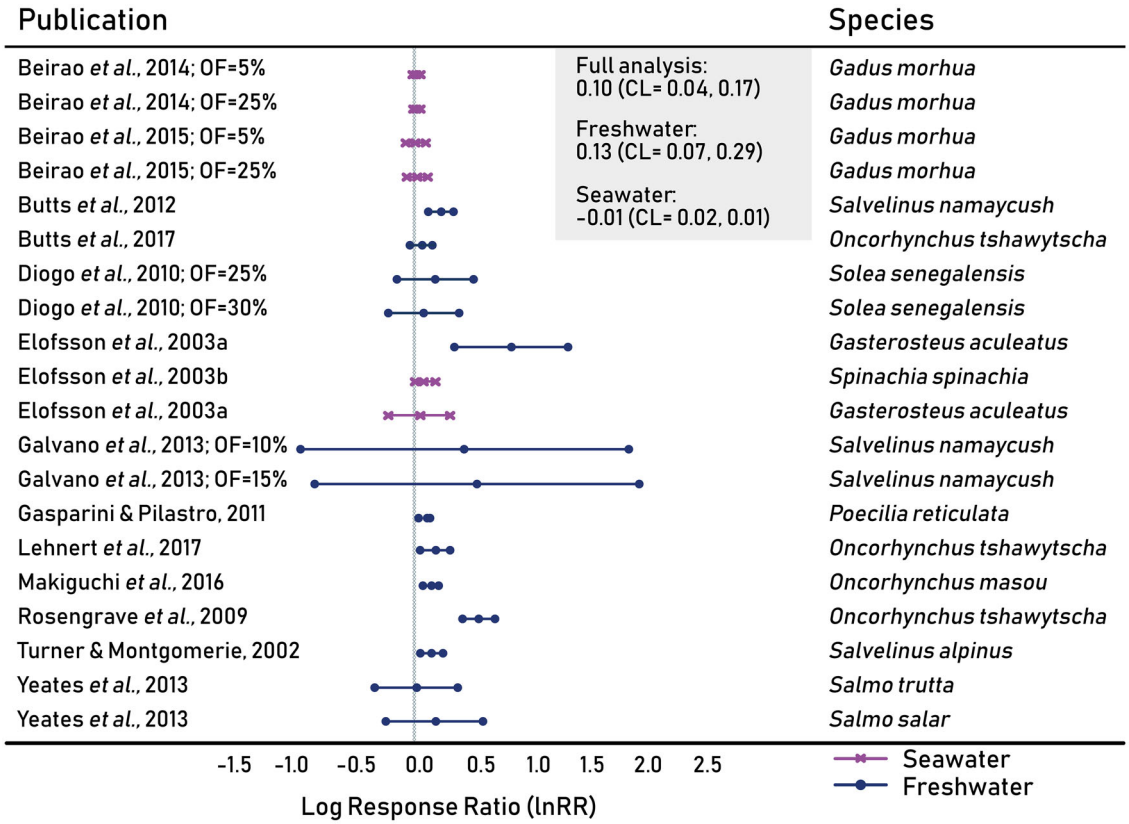

Figure 6. Forest plot for the effects of ovarian fluid (OF) on sperm velocity (n = 20 effect sizes). Study ID is given on the left and species of fish used in each is listed on the right. Effect sizes are given as log response ratios (lnRR) with 95% confidence limits for all species. Effect sizes with confidence intervals that do encompass 0 are not statistically significant.

mild correlations were detected among related species in the effects of OF on sperm velocity but not for motility. Future work on under-represented species groups could further clarify the impacts of phylogeny on sperm motility and velocity in response to OF.

This analysis did not detect any significant effect of OF concentration on overall effect magnitude, but reports from within individual studies indicate that this effect can be quite apparent. For sperm activation studies in the laboratory, there is diverse variation in how much OF is included in the activation medium (as little as 5% to as high as 100%) based on the species of interest and their respective reproductive strategies. Interestingly, in some fish species, OF causes either a negative or neutral impact on sperm performance (Wojtczak et al. 2007; İnanan and Öğretmen 2015; Kleppe et al. 2018). which may be partly due to OF concentration. Negative relationships may be observed with high OF concentrations because of inhibition of the osmotic mechanisms needed for sperm activity and increased viscosity of the activation media (Beirão et al. 2014), but the opposite effect has also been documented. In one example, sperm velocity was enhanced at 50% OF but inhibited at 5% (Turner and Montgomerie 2002). In another example, there was no difference in sperm performance between 5% and 25% OF (Beirão et al. 2014), indicating that the differences in concentrations may not have been high enough to observe an inhibition effect. The inability to detect significance for OF concentration as a moderator is likely due to such conflicting reports between studies, a lack of standardization in activation protocols across species, and differences between concentrations that were compared.

This analysis comprised primarily of salmonids, in which high positive effect sizes have been reported. The spawning behavior of salmonids may have encouraged sperm to react positively to OF over evolutionary history. Males and females gather in huge aggregations to spawn in heavily crowded areas, introducing fierce male competition at the gametic level (Mjølnerød et al. 1998). Sperm must have high motility and velocity -traits that were enhanced by OF in the studies we analyzed. One reason may be due to the high percentages of OF expelled with the eggs compared to other families, which can be 10-30% volume relative to the egg mass (Lahnsteiner et al. 1999; Wojtczak et al. 2007). As mentioned

previously, OF plays many crucial roles during the fertilization process and has been shown to have a necessary function in enhancing fertility specifically for this family (Urbach et al. 2005; Galvano et al. 2013).

Another source of variation may be related to the population origin of the fish, and/or genetic relatedness between populations (Beirão et al. 2014,2015). This hypothesis is clearly validated in distinct Atlantic cod, where OF from southern origin females had greater inhibiting effects on sperm performance for northern males than those from their native population, indicating a preference for mates within the same population (Beirão et al. 2015). There is evidence that fish OF composition shows intra-species variation, particularly with respect to the constituents known to influence sperm behavior (Lahnsteiner et al. 1995; Wojtczak et al. 2007; Rosengrave et al. 2009b). Therefore, OF composition between females of each population likely resulted in differential sperm performance among genetically diverged populations and acted as a method of reproductive isolation in some marine species such as Atlantic cod. Further evidence of intraspecific variation in sperm-OF interactions was also indicated in freshwater species. For example, sperm from genetically unrelated males was estimated to be 10% more competitive in fertilization trials with OF than their relatives in guppies (Gasparini and Pilastro 2011). This relationship shows the opposite effect by selecting against more genetically similar individuals. Within the same population individuals are not affected by reproductive isolation, so it may serve to prevent inbreeding at the gametic level. It has been proposed that cryptic female choice may be a significant selection mechanism responsible for this variation within and across populations (Gasparini and Pilastro 2011; Mautz et al. 2013; Yeates et al. 2013). Whether or not the negative responses in our analysis were due to population-level mate preference certainly warrants further study. Due to a small number of studies, this effect was unable to be properly examined as a source of heterogeneity in our meta-analysis.

Freshwater and saltwater environments each present unique challenge that sperm must overcome to reach the egg and achieve fertilization, altering characteristics of sperm motility and longevity. To fully explain the patterns in our results, we must be aware of the many different properties that distinguish each activation environment such as the drastic differences in osmolality and salinity. For instance, one notable difference is that the ions that comprise the OF micro-environment may be altered by the osmolality of the external environment (Hirano et al. 1978; Elofsson et al. 2006). Thus, sperm are responding to different biochemical cues. Freshwater and saltwater differ tremendously by ion composition, which is shown to alter sperm performance, especially in freshwater fishes (Alavi and Cosson 2006). Therefore, it is possible that sperm of freshwater fishes show higher positive responses when activated in OF because maternal ions that stimulate sperm activity are more easily detected in a low-ion environment (Elofsson et al. 2006; Rosengrave et al. 2009a). This effect would not be so apparent in marine species due to high amounts of ions already present that interfere with the sperm's ability to distinguish and respond to the OF micro-environment. Due to the shorter duration of motility and more immediate need to reach an egg in a shorter window of time for freshwater species (Kime et al. 2001; Alavi et al. 2007), sperm of freshwater fish may be more responsive to organic constituents (e.g. proteins, metabolites, enzymes) as well, although the mechanisms behind these processes require further research.

Furthermore, the timing of activation trials could have led to some significant results going undetected. In some cases, sperm may show a delayed response that occurs beyond 0-15 s post activation. For example, OF did not yield a positive response until 30 s post-activation for Caspian brown trout, *Salmo trutta caspius* (Hatef et al. 2009). Furthermore, in sticklebacks (in which sperm motility can last several minutes), there were positive responses for sperm velocity activated in seawater after 45 min (Elofsson et al. 2003a) and after 60 min post-activation (Elofsson et al. 2003b). With activation times in our analysis limited to the first 0-15 s, any delayed responses were not considered, but it is unlikely that they are relevant to a male's fertilization success (Casselman et al. 2006). To illustrate the importance of immediate fertilization, a 2 s delay of sperm release caused significant reductions of paternity in Atlantic salmon (Yeates et al. 2007). Furthermore, in sockeye salmon, *Oncorhynchus nerka*, 80% of fertilization was achieved within first 5 s of sperm-egg mixing (Hoysak and Liley 2001) These results suggest that sperm swimming speed is an important trait of male gamete quality, and even small variations in the timing of gamete interactions could have consequences for fertility.

Lastly, this analysis also points to differences in methodology between studies that are not held constant (i.e. methods of sperm extraction/processing and activation techniques) that may have contributed underlying effects. Subtle differences such as sperm

density, seminal plasma and OF pH, and dilution rates varied between studies and were sometimes not even reported, which could have altered sperm swimming behavior. OF concentration also ranged widely from low to high across studies. Although methodology was not analyzed statistically because of a small number of studies for both traits, it was still likely to be a large source of remaining unexplained heterogeneity in our analysis. Future studies may confirm or expand off our interpretation of the results as more data becomes available and methodology becomes more consistent.

Conclusion

Results of this meta-analysis showed that sperm velocity was enhanced by the presence of female OF for species spawning in freshwater environments. Because no such relationship was found for the marine species in our analyses, it was determined that spawning environment can have significant impacts on the magnitude and direction of the observed effects. Results are also subject to vary based on phylogeny of the fish species. We are confident that our investigation was a reliable method that allowed for representative quantification of the overall true effect and explore sources of variability across studies. We limit our interpretation to the range of species on which data are available, comprising primarily of species sharing similar phylogeny and lineage. With more research done on OF as a factor to impact fertility across a wider range of species, it may serve to broaden the understanding of natural processes that govern sperm performance, reproductive success across fish species, and fertilization techniques in hatcheries.

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