

Sperm adaptation in relation to salinity in three goby species

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Abstract

In externally fertilizing species, the gametes of both males and females are exposed to the influences of the environment into which they are released. Sperm are sensitive to abiotic factors such as salinity, but they are also affected by biotic factors such as sperm competition. In this study, the authors compared the performance of sperm of three goby species, the painted goby, *Pomatoschistus pictus*, the two-spotted goby, *Pomatoschistus flavescens*, and the sand goby, *Pomatoschistus minutus*. These species differ in their distributions, with painted goby having the narrowest salinity range and sand goby the widest. Moreover, data from paternity show that the two-spotted goby experiences the least sperm competition, whereas in the sand goby sperm competition is ubiquitous. The authors took sperm samples from dissected males and exposed them to high salinity water (31 PSU) representing the North Sea and low salinity water (6 PSU) representing the brackish Baltic Sea Proper. They then used computer-assisted sperm analysis to measure the proportion of motile sperm and sperm swimming speed 10 min and 20 h after sperm activation. The authors found that sperm performance depended on salinity, but there seemed to be no relationship to the species' geographical distribution in relation to salinity range. The species differed in the proportion of motile sperm, but there was no significant decrease in sperm motility during 20 h. The sand goby was the only species with motile sperm after 72 h.

KEYWORDS

Gobiidae, *Gobiusculus*, salinity, sperm competition, sperm longevity, sperm motility

1 | INTRODUCTION

To ensure maximal fertilization success, sperm need to be adapted to both abiotic and biotic factors. In external fertilizers, this means that sperm must survive and perform in the aquatic environment into which they are released during mating. Salinity is a key abiotic factor affecting the viability and function of sperm in many organisms including echinoderms (Allen & Pechenik, 2010), amphibians (Byrne *et al.*, 2015) and fish (Elofsson *et al.*, 2003a; Elofsson *et al.*, 2003b;

Nissling *et al.*, 2002; Nissling & Larsson, 2018; Nissling & Westin, 1997; Svensson *et al.*, 2017), and can limit the distribution of a species (Alavi & Cosson, 2006; Byrne *et al.*, 2015; Green *et al.*, 2020; Svensson *et al.*, 2017). In species with a geographical distribution covering a range of salinities, or for estuarine and intertidal species that routinely experience salinity fluctuations, individuals can be expected to show generally broad salinity tolerances (Tiersch & Yang, 2012; Yang & Tiersch, 2009). Conversely, if gene flow is restricted, subpopulations may be locally adapted to prevailing salinity

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levels (DeFaveri & Merilä, 2014; Serrao *et al.*, 1996; Svensson *et al.*, 2017).

Sperm adaptation is also strongly affected by a species mating system. Sperm competition occurs when sperm from two or more males simultaneously try to fertilize the same group of eggs (Parker, 1970), and it is common in species with both internal and external fertilization (Birkhead & Møller, 1998; Fitzpatrick, 2020). When sperm competition is common, it is expected that selection will promote sperm traits that improve fertilization success, such as increased longevity after release (Birkhead & Møller, 1998; Snook, 2005). The authors of this study therefore might expect that in systems where risk for sperm competition *via* parasitic spawning (*i.e.*, sneaking and other nest intrusions aiming to fertilize eggs; Taborsky, 1994) is high, not only sperm expenditure (Stockley *et al.*, 1997), but sometimes also sperm longevity should be higher (Fitzpatrick *et al.*, 2009). Experimental and observational results on this are, however, somewhat variable (Morita *et al.*, 2014), and recent studies show that different sperm motility traits could determine fertilization success (Lymbery *et al.*, 2018).

In this study, the authors compare sperm performance (proportion of motile sperm and sperm swimming speed) with respect to salinity and levels of sperm competition in three different species of gobies, the painted goby, *Pomatoschistus pictus* (Malm, 1865), the two-spotted goby, *Pomatoschistus flavescens* (Fabricius, 1779), and the sand goby *Pomatoschistus minutus* (Pallas, 1770). Of the three, the painted goby has the most restricted salinity distribution and is found in full salinity waters from the Mediterranean to the North Sea but is not found east of Öresund in the brackish waters of the Baltic Sea. The two-spotted goby is similarly distributed along the coasts of the North Sea and also extends into the Baltic Sea to the south coast of Finland where the salinity is only 5–6 PSU. The sand goby exhibits the widest geographical distribution in relation to salinity. It occurs and breeds along most of the European coastline, from the Mediterranean Sea to northern Norway, and the entire brackish water of the Baltic Sea, including its northernmost reaches (Bothnian Bay) where salinity is only 3–5 ppm (Kullander *et al.*, 2012; Miller, 1986). The sand goby also reproduces in estuaries and the tidal zone where it can be exposed to a range of salinities (Miller, 1984).

All three study species use a range of nesting substrates to which females attach eggs. The sand and painted gobies build nests by excavating cavities with a single nest opening underneath empty bivalve shells, which they also cover with sand leaving a single narrow nest opening (Bouchereau *et al.*, 2003; Hesthagen, 1979). In contrast, the two-spotted goby does not cover its nest and often has a completely open nest in an empty shell or a fold of macro algae (Amundsen, 2018). The male remains with the nest and guards and cares for the eggs until hatching. Based on DNA analysis of eggs from nests collected in the field, it is known that multiple paternity is relatively rare in the two-spotted goby (Mobley *et al.*, 2009; Monroe *et al.*, 2016), but common in the sand goby (Jones *et al.*, 2001a; Jones *et al.*, 2001b), indicating a higher risk of sperm competition for nest holding males of the latter species. For the painted goby, the corresponding information does not yet exist.

Upon release, sperm are activated by physiological reactions initiated by the surrounding medium, often a change in osmolality, which is largely determined by salinity (Alavi & Cosson, 2006). Given the large differences in geographical range among these three goby species, this study's authors hypothesize that sperm performance in different salinities will depend on the range of salinities encountered in a species' natural geographical distribution. In this study, they test this prediction, predicting the painted goby to be least tolerant to salinity variation, the two-spotted goby intermediate and the sand goby to be most tolerant to salinity variation. Furthermore, it is predicted that sperm velocity and longevity will be affected by risk of sperm competition (Liao *et al.*, 2018). Based on existing information regarding multiple paternity for two of the three species, the authors therefore predict that sand gobies have faster swimming and longer living sperm than two-spotted gobies.

2 | MATERIALS AND METHODS

All fish were caught at the Swedish west coast close to the Kristineberg Marine Research Laboratory, 28–29 May 2012. Sand gobies and painted gobies were caught from shallow and sandy areas (Bökevik and Smalsund) using a beach seine, whereas two-spotted gobies were caught from a habitat with more macroalgae (next to Rättholmen) using hand-nets while snorkelling. Natural salinity in this area varies, but a long-term average, measured within 0–20 m, is 26.2 PSU (min–max 7.3–34.5 PSU) (Table S1 in Leder *et al.*, 2021).

The fish were sorted according to sex and species and kept in 50 l storage tanks overnight, before they were used in the study. All storage tanks had a through-flow of seawater pumped directly from the adjacent bay at a depth of 7 m, with a temperature of $13.5 \pm 1.7^\circ\text{C}$ (mean \pm S.D.) and salinity of 23.0 ± 3.1 PSU. The bottom of each tank had a 3 cm layer of sand for the fish to burrow in. The fish were fed a mix of frozen chopped mussel meat, brown shrimp and Alaska Pollock.

Eight painted goby males, seven two-spotted goby males and eight sand goby males, all in breeding colouration, were included in the study. Each male was decapitated, pithed and dissected immediately thereafter. From each male a sperm sample was obtained for analysis of proportion of motile sperm and sperm velocity (swimming speed) immediately after sampling, and for analysis of longevity (proportion of motile sperm after 20 h), in high and low salinity, as described below.

Before dissection, two tubes were prepared for each male, with 750 μl of either high salinity water (31 PSU, made from filtered natural sea water, pumped from 32 m depth) representing the North Sea or low salinity water (6 PSU, made from the same water as above, but diluted with distilled water to the desired salinity) representing the brackish Baltic Sea Proper. These tubes were kept in a rack surrounded by water of $14.0 \pm 0.5^\circ\text{C}$.

The motility assay followed that of Havenhand and Schlegel (2009). Briefly, one testis was placed in an Eppendorf tube, the tissue was ruptured using a scalpel and 20 μl of Atlantic cod

calcium-free Ringer's solution at pH 7.5 (150 mM NaCl, 5.2 mM KCl, 1.8 mM MgSO₄, 7.0 mM NaHCO₃, 1.9 mM NaH₂PO₄; Karila *et al.*, 1993) was added. The tube was closed and shaken quickly by hand. From this solution, 8 μ l was suspended into the tube with high salinity water and 8 μ l into the tube with low salinity water. The suspension was mixed by turning the tubes upside down twice. A 40 μ l drop of the sperm suspension was placed between an albumin-coated microscope slide and coverslip, separated by a 0.75 mm thick O-ring.

Sperm movement was recorded for 0.5 s at the midpoint of the drop, at 30 frames per second, using a digital video camera (Pixellink 700 series, PixelLink) mounted on an inverted microscope (Leica DM-IL, Leica Microsystems). Pilot experiments showed that illumination by the microscope lamp had no impact on the temperature inside the drop during videotaping (time of slide on microscope: c. 10 s). Nine technical replicates were taken from each combination of species and salinity, always with three samples on each of three microscope slides. All recordings were performed within 30 s after placing the sample on the slide, and within 10 min of dissection.

Pilot tests indicated that for all three species sperm longevity was exceptionally long. To assess this, the authors resampled each tube with three to six 40 μ l technical replicates per male and salinity after c. 20 h. They still had sufficient sperm suspension left in a few tubes for a third sampling after 72 h. Despite this data set being incomplete, these results are included in the current study to illustrate an exceptionally long sperm longevity found in one of the species. The tubes with sperm suspensions were kept in $14.0 \pm 0.5^\circ\text{C}$ water between the sampling times.

Videos were post-processed and analysed with ImageJ using the computer-assisted sperm analysis (CASA) plugin (Wilson-Leedy & Ingermann, 2007). From the videos (see species-specific CASA settings in Supporting Information Table S1), the authors determined the proportion of motile sperm relative to the total number of sperm in the video clip. To exclude non-swimming particles drifting in micro-currents, sperm were classified as swimming only if velocity was higher than $15 \mu\text{m s}^{-1}$. The authors also determined the swimming speed of the sperm, measured as the curvilinear path velocity (VCL), the straight line velocity (VSL) and the average path velocity (VAP). Data for these metrics were highly correlated (all $r > 0.935$, $P < 0.001$), and therefore the authors only include analyses of VCL below. VCL was chosen as it is a standard metric for measuring sperm swimming speed as this best represents the speed of sperm along the swimming path and is a close proxy for fertilization success (Gage *et al.*, 2004; Purchase & Moreau, 2012). Longevity was measured as proportion sperm that were motile (moving faster than $15 \mu\text{m s}^{-1}$) after 10 min, 20 and 72 h.

The design to study the effect of salinity on the proportion of motile sperm and VCL follows a repeated measures design because individual sperm samples were divided between the two salinity treatments (high and low). Therefore, salinity was the within subjects factor, and species was the between subjects factor. For the analysis of the proportion of motile sperm, the authors summed up the number of motile sperm in the technical replicates to provide the target variable and the total number of sperm in the technical replicates to provide the number of trials variable. The authors then used a generalized linear mixed model with a binomial probability distribution

and logit link function to analyse the proportion of motile sperm. VCL was averaged over the technical replicates and then analysed using generalized linear mixed model with a gamma probability distribution with log link function.

The effect of time (longevity) on the proportion of motile sperm and VCL for the three species was analysed using a repeated measures design with times 10 min and 20 h as the within subjects factor and species as the between subjects factor. The proportion of motile sperm was analysed using a generalized linear mixed model with a binomial probability distribution and logit link function following the procedure described above. VCL was analysed using generalized linear mixed model with a gamma probability distribution with log link function.

For all analyses, the authors used Satterthwaite approximation for degrees of freedom because they had different sample sizes for the different treatment groups. All analyses were performed in SPSS (v 26).

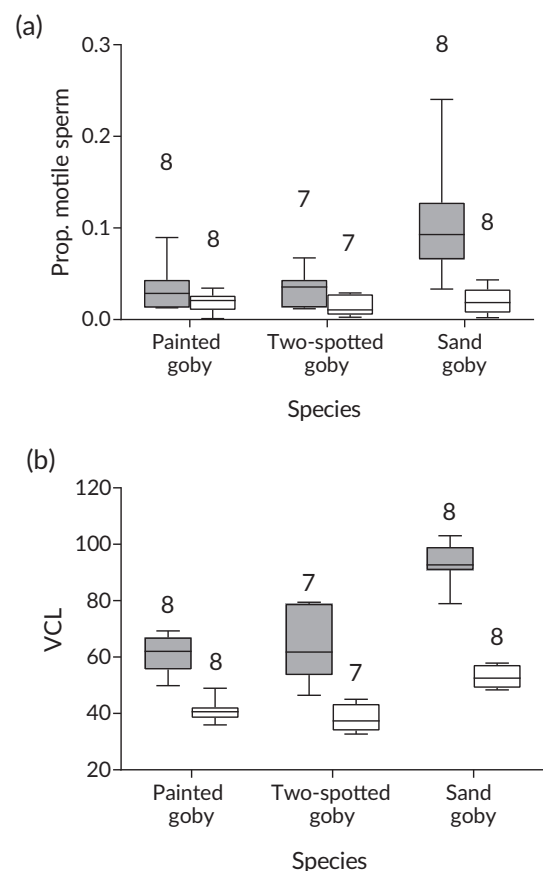


FIGURE 1 Box and whisker plots of sperm motility and swimming speed in relation to salinity in the painted goby (*Pomatoschistus pictus*), two-spotted goby (*Pomatoschistus flavescens*) and sand goby (*Pomatoschistus minutus*). (a) Proportion of motile sperm after 10 min for the three goby species in full marine water (31 PSU, white bar) and diluted marine water (6 PSU, grey bars). (b) Curvilinear path velocity (VCL) after 10 min for the three goby species in full marine water (white boxes) and diluted marine water (grey boxes). The box encloses the 25th and 75th percentiles with the median indicated by the horizontal line. The whiskers indicate minimum and maximum values. Numbers above boxes indicate sample sizes

2.1 | Ethical statement

The number of fish killed was kept to a minimum. This study was performed under Swedish ethical permits 135-2010 and 143-2012.

3 | RESULTS

3.1 | The effect of salinity

The response to salinity in the proportion of motile sperm differed significantly among species (generalized mixed linear model: species by salinity interaction effect, $F_{2,32} = 4.76$, $P = 0.016$). In all species, the proportion of motile sperm was higher in 31 PSU (salinity effect, $F_{1,31} = 16.78$, $P < 0.001$; Figure 1a), and this effect was biggest in the

sand goby (Figure 1a) creating the above-mentioned interaction and resulting in significant differences among the species (species effect, $F_{2,32} = 7.76$, $P = 0.001$). The proportion of motile sperm in painted and two-spotted goby was very similar (for model parameter estimates, see Supporting Information Table S2).

The three species exhibited a difference in curvilinear path velocity (species effect, $F_{2,37} = 45.50$, $P < 0.001$) with the sand goby having the fastest-moving sperm (Figure 1b). Salinity again had a strong and similar effect on velocity in all three species (salinity effect, $F_{1,37} = 202.70$, $P < 0.001$; Figure 1b). There was no significant interaction effect, $F_{2,37} = 2.02$, $P = 0.15$; for model parameter estimates, see Supporting Information Table S3).

3.2 | Sperm longevity

There were large differences among the species in the overall proportion of motile sperm (species effect, $F_{2,30} = 17.45$, $P < 0.001$; Figure 2a). All three species had motile sperm after 20 h, but there was no significant effect of time on the proportion of motile sperm (time effect, $F_{1,31} = 0.97$, $P = 0.332$; interaction effect, $F_{2,30} = 1.95$, $P = 0.160$; Figure 2a). In the sand goby, live sperm could still be observed after 72 h, although the proportion of motile sperm was markedly lower than after 10 min and 20 h. No two-spotted or painted goby sperm were motile at that point, although the sample sizes for these species were very small (Figure 2a; for model parameter estimates, see Supporting Information Table S4).

The curvilinear path velocity differed among the species (species effect, $F_{2,29} = 19.36$, $P < 0.001$; Figure 2b), but there were no significant effects of time ($F_{1,29} = 3.22$, $P = 0.083$) or of the interaction between species and time ($F_{2,29} = 0.33$, $P = 0.724$; Figure 2b; for model parameter estimates, see Supporting Information Table S5).

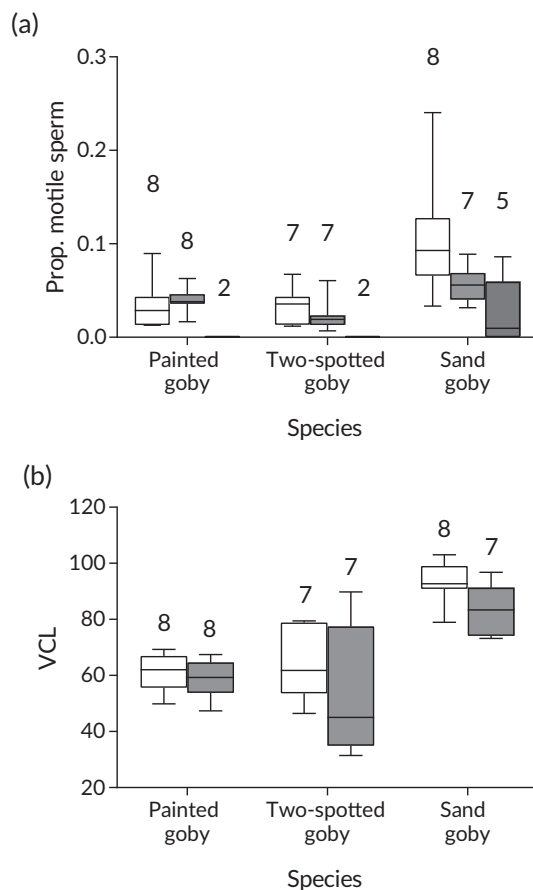


FIGURE 2 Box and whisker plots of longevity of sperm in the painted goby (*Pomatoschistus pictus*), two-spotted goby (*Pomatoschistus flavescens*) and sand goby (*Pomatoschistus minutus*). (a) Proportion of motile sperm after 10 min (white boxes), 20 h (grey boxes) and 72 h (dark grey boxes) for the three goby species in full marine water (31 PSU). (b) Curvilinear path velocity (VCL) after 10 min (white boxes) and 20 h (grey boxes) for the three goby species in full marine water (31 PSU). The box encloses the 25th and 75th percentiles with the median indicated by the horizontal line. The whiskers indicate minimum and maximum values. Numbers above boxes indicate sample sizes

4 | DISCUSSION

The authors found clear differences in sperm motility and velocity among the three goby species. The sand goby tended to have the highest values for all traits, especially in high salinity, which is the salinity level most similar to the marine location from which they were caught. It is especially noteworthy that for all three species the proportion of motile sperm showed no or very slight decrease after 20 h. Even after 72 h, the authors found live sperm in the sand goby. In most fish sperm are reported to have a much shorter longevity, on the order of seconds to tens of minutes (Browne *et al.*, 2015), but gobies appear to be an exception. For example, in the marine dusky frillgoby, *Bathygobius fuscus* sperm have been reported to have longevities of nearly 10 h (Nakanishi & Takegaki, 2019), and sand goby sperm still alive after 24 h has been documented using live/dead cell staining techniques (Green & Kvarnemo, 2019).

The sand goby has the widest distribution occurring along Europe's coast (Miller, 1986) including full marine and low salinity areas. Low salinity areas include estuaries and the brackish Baltic Sea,

where it occurs and breeds in near fresh water (Wiederholm, 1987). If the range of salinities encountered by a species is reflected in the range of salinities its sperm can tolerate, then one would expect the sand goby to show sperm traits that are least affected by the salinity treatment. Similarly, the painted goby should exhibit the highest sensitivity to salinity because it has the most narrow salinity distribution, whereas the two-spotted goby should be intermediate (Kullander *et al.*, 2012). Nonetheless, the results of this study indicate that there are no clear differences among the species in the degree to which salinity tolerance is related to the salinity ranges covered by the species distribution. All three species showed a higher proportion of motile sperm and higher sperm velocity in the high salinity treatment. A possible explanation for this result is that the activation of sperm is a plastic trait, as found, *e.g.*, in three spine stickleback, *Gasterosteus aculeatus* Linnaeus, 1758, in which the developmental environment determines the conditions under which sperm are activated (Taugbol *et al.*, 2017). The authors of this study therefore suggest that in the two species (sand and two-spotted goby) that have invaded low salinity environments from a marine ancestry, the sperm responses to salinity have evolved through local adaptation rather than facilitated through exaptation. For the sand goby there is further evidence to support this conclusion, as males sampled from a low salinity site (3 PSU at Hårnösand in the Bothnian Bay) showed markedly better sperm performance in low than in high salinity (Leder *et al.*, 2021; Svensson *et al.*, 2017) and show strong genome differentiation along the salinity gradient between these sites (Leder *et al.*, 2021).

Sticklebacks are an interesting group of fish for comparison to the authors' findings on the gobies. Three spine sticklebacks are widely distributed, in both marine and freshwater habitats. Three spine sticklebacks that are sympatric to the gobies studied here show much shorter sperm longevity in low salinity (Elofsson *et al.*, 2003a). The 15 spine stickleback, *Spinachia spinachia* (Linnaeus, 1758), extensively overlaps the distribution of the two-spotted goby and is similarly limited by minimum salinity (Gross, 1978). Fifteen spine stickleback sperm are more sensitive and short-lived in brackish water (Elofsson *et al.*, 2003b) than three spine stickleback sperm. The authors found the same pattern in the gobies, the species with a more salinity restricted distribution showing more limited sperm survival at lower salinities.

Salinity tolerance of sperm is likely to reflect a species' ability to invade novel environments (Green *et al.*, 2020), and the fertilizing ability of sand goby sperm is a function of a male's native salinity (Svensson *et al.*, 2017). In the current study, the proportion of motile sperm was always lower in the low than the high salinity treatment, suggesting that the sperm performance of all three species was indeed better adapted to high salinity. Given that all three species are of marine origin, and the sampled fish came from a marine site, this is perhaps not surprising. It is thought that sperm of marine fish species are activated upon exposure to a hyperosmotic medium (Morisawa, 1985), but in some cases, it has been shown that the life span of sperm is longer if the water is slightly diluted as compared to water of native salinity (Groison *et al.*, 2010; Lahnsteiner & Patzner, 1998). The authors found

no indication of such an effect as in all cases motility and speed were lower in the low salinity treatment, but the experiment of this study was not designed to test this particular observation.

In gobies sperm competition is often manifested as multiple paternity arising from parasitic spawning in a single male's nest (Jones *et al.*, 2001b). The authors found that the sand goby had the highest sperm motility and velocity (and sperm still alive after 72 h), traits that are argued to be shaped by sperm competition (Fitzpatrick, 2020; Snook, 2005). Parasitic spawning is common in the sand goby (Jones *et al.*, 2001b; Singer *et al.*, 2006). In contrast, the two-spotted goby had much lower sperm motility and velocity, and this species is known to have very low frequencies of multiple paternity suggesting low sperm competition levels (Mobley *et al.*, 2009; Monroe *et al.*, 2016). Therefore, the ejaculate traits found in this study support the expectation of sperm competition theory and the authors' predictions. A definitive weakness of this study is the lack of information about multiple paternity frequencies in the painted goby. Nonetheless, based on the sperm trait findings of this study resembling the two-spotted goby, the authors predict that parasitic spawning and sperm competition in this species are relatively rare.

Another possible reason for long-lived sperm is a need to fertilize eggs over an extended time (Scaggiante *et al.*, 1999). Egg laying in gobies generally is a slow process where the female attaches her eggs to the nest substrate one at a time, sometimes resulting in a total spawning time of several hours (Marconato *et al.*, 1996). For example, in the sand goby a female needs an hour or more to deposit her egg clutch (CK, KL, OS, pers. obs.). The sand and painted gobies build closed cavities as nest sites, which may aid in keeping the sperm near fertilizable eggs for an extended time as compared to free spawning species (Liao *et al.*, 2018; Morita *et al.*, 2014) or species spawning in turbulent water (Neat *et al.*, 2003). A successful sand goby male can mate with several females in sequence (Jones *et al.*, 2001b). In a closed nest cavity, a single long-lived sperm ejaculation could remain in the nest and fertilize eggs from several females. Recently, in the dusky frillgoby with similar nesting behaviour as the sand goby, nest guarding males were shown to use tail fanning to remove sperm from other males (Takegaki *et al.*, 2020). Furthermore, in many gobies males prepare the spawning surface with a sperm containing mucus trail from which new sperm are activated over time, as the mucus dissolves (Marconato *et al.*, 1996; Scaggiante *et al.*, 1999; Svensson & Kvarnemo, 2005), creating a further reason for increased longevity of sperm. If extended sperm longevity is adaptive, then the authors predict that it will be correlated with the nest structure and the mating system of the species. Specifically, in mating systems where successful males spawn with many females sequentially, and in relatively enclosed microenvironments, the authors expect sperm longevity to be extended (Morita *et al.*, 2014).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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