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# Environmental salinity-induced shifts in sperm motility activation in *Fundulus grandis*

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ABSTRACT

Motility activation of fish sperm typically responds to levels of specific ions or osmotic pressure differences between the surrounding water and body tissues. In general, the sperm of marine fishes are activated by an increase in osmotic pressure (hypertonic salinity), and that of freshwater species by a decrease (hypotonic salinity). These stenohaline species exist in relatively stable environments, however, estuarine fishes are exposed to rapidly changing and broad salinity ranges, often resulting in external osmotic pressures that include those of the body (isotonic). To assess the ability of *Fundulus grandis* sperm to adapt to changes in salinity, adult males were acclimated to salinities of 0, 5, 10, 20, 35, or 50 ppt and held for 30 d. The testes were dissected from the fish and sperm were activated with deionized water, various osmolalities (100–1000 mOsmol/kg) of Hanks' balanced salt solution (HBSS), calcium-free HBSS ($Ca^{2+}$-Free HBSS), and sodium chloride solution (NaCl). The deionized water did not activate sperm motility regardless of the acclimated salinity. Compared to HBSS, $Ca^{2+}$-Free HBSS and NaCl activated sperm motility with a significantly lower percentage at the same osmolalities. The osmolality eliciting the highest motility activation was significantly different ($P<0.01$) among acclimated groups and shifted from 300 mOsmol/kg (ranging from 200 to 500) for sperm collected from 5 ppt, 500 mOsmol/kg (ranging from 200 to 800) for sperm collected from 10 ppt, 600 mOsmol/kg (ranging from 400 to 700) for sperm collected from 20 ppt, 800 mOsmol/kg (ranging from 200 to 900) for sperm collected from 35 ppt, and 900 mOsmol/kg (ranging from 600 to 1000) for sperm collected from 50 ppt. Motility peaked after 30 s exposure to HBSS, and decreased over 10 min. Motility exhibited a similar initial pattern when exposed to $Ca^{2+}$-Free HBSS, however, the sperm gained motility at lower osmolalities over 10 min, exhibiting multiple peaks. These results indicate that environmental salinity can significantly influence sperm behavior in adult males of *F. grandis* with substantial changes after only 30 d of acclimation. As such, this should be considered as a major unrecognized variable in sperm research in this species and can be considered for use in optimizing protocols addressing in-vitro fertilization and cryopreservation. Whether this phenomenon is unique to *Fundulus* or is a characteristic of euryhaline fishes remains unresolved.



## 1. Introduction

*Fundulus grandis* is a euryhaline fish with a natural distribution extending from Vera Cruz, Mexico, along the US Gulf coast, to the northern Florida Atlantic coast (Wallace and Waters, 2004). The species is sexually dimorphic with females exhibiting a uniform, dull grey to silver coloration, and males with golden opercula, dark spotting along the lateral area, and vibrant, dichroic scales and fins (Wallace and Waters, 2004). The habitat of *F. grandis* is typically within coastal estuary systems in waters ranging from fresh to hypersaline (salinity of more than 35 ppt which is full-strength sea water) (Kilby, 1955; Renfro, 1960). These systems are subject to tidal fluxes, exposing fish to rapid and sometimes wide changes in salinity. Although this species is tolerant of fresh water for short periods, the limit of exposure is one month (Griffith, 1974). The tolerable salinity range for osmotic and ionic regulation is from 2 to 70 ppt (Nordlie and Haney, 1998).

This species is capable of reproduction year round (Kilby, 1955; Renfro, 1960), and is present throughout estuary systems which makes it a commonly used baitfish in the southeastern United States. Generally females can only produce 10–20 eggs per spawn, but they spawn repeatedly during the season by depositing eggs on vegetation (Wallace and Waters, 2004). Fertilization usually occurs immediately after spawning by attendant males. Eggs can tolerate exposure to air for 12–18 d and can hatch when returned to the water. However, little is known about sperm behavior and larval development. There are about 40 species in the genus *Fundulus*. Most are small (less than 10 cm in body length) and estuarine (www.fishbase.org), and thus are candidates for use as research models (Roszell and Rice, 1998; Williams et al., 2010). Due to its widespread distribution along the

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eastern coastal marshes of US, and some inland systems, a single species *Fundulus heteroclitus* has become an important research model for toxicology, osmotic regulatory physiology, and ecological and evolutionary genetics (see comprehensive review Burnett et al., 2007). Interest in genomic research of this genus is also rapidly developing because of its wide use in research (www.ncbi.nlm.nih.gov).

Gamete activation in fish is a necessary step during artificial spawning and other experimental manipulations involving fertilization. Generally, the sperm of most fish are quiescent in the testes, and their activation relies on the difference in osmotic pressure or ion levels between the testicular fluid and the outside environment (Alavi and Cosson, 2006; Coward et al., 2002), but can be influenced by other factors such as pH and temperature (Alavi and Cosson, 2005). Specifically, four different modes have been categorized: 1) for most fishes with external fertilization, sperm motility can be initiated by either hypotonic (for freshwater fish) or hypertonic osmolalities (for marine fishes). Once activated, the sperm generally have a short period of motility (30 s–5 min) (Cosson, 2004; Morisawa and Suzuki, 1980; Morisawa et al., 1983; Yang et al., 2007). 2) Sperm motility can also be initiated by alteration of the concentration of certain ions, such as in salmonid fishes, where motility can be initiated by reducing the concentration of potassium ion (Cosson, 2004; Morisawa and Suzuki, 1980; Morisawa et al., 1983). 3) For live-bearing fishes, such as those of the freshwater genus *Xiphophorus* (Yang et al., 2006) and the marine ocean pout *Macrozoarces americanus* (Wang and Crim, 1997; Yao et al., 2000), sperm motility can be initiated by isotonic osmolalites (around 300 mOsmol/kg), but not by hypotonic or hypertonic osmolalities. Once initiated, the sperm of these species can remain continuously motile for as long as one week (Huang et al., 2004; Yang et al., 2006). And, 4) for euryhaline fishes, sperm can be activated across a wide range of osmolalities. Previous research in euryhaline fishes has been reported in medaka *Oryzias latipes* (Yang and Tiersch, 2009b), the tilapia species *Oreochromis mossambicus* (Linhart et al., 1999; Morita et al., 2003, 2004) and *Sarotherodon melanotheron* (Legendre et al., 2008). The results for tilapia suggested that motility activation of sperm of euryhaline fishes could be affected by the environmental salinity. Thus, investigation is needed using other euryhaline fish species to ascertain the effect of environmental salinity on sperm behavior.

The goal of this study was to characterize the motility activation mode of sperm from *F. grandis* and the effect of environmental acclimation salinities. The objectives were to evaluate the: 1) activation of sperm motility at osmolalities ranging from deionized water, to 100–1000 mOsmol/kg of HBSS, $Ca^{2+}$-free HBSS, and NaCl, 2) effect of 30-d acclimation at environmental salinities from 0 to 50 ppt on sperm motility activation, and 3) effect of the presence or absence of calcium ions on motility activation. The results of this study provide initial insight on the mechanism of motility activation in euryhaline fishes, could facilitate sperm cryopreservation for which knowledge of motility activation is necessary, could improve artificial fertilization manipulation in this species, and could be representative of other *Fundulus* fishes, especially *F. heteroclitus* which is becoming an important research model.

## 2. Materials and methods

### 2.1. Fish

Male *F. grandis* were purchased from a local farmer in Thibodaux, Louisiana where they were maintained at a salinity of 10 ppt. Fish were brought to the Aquaculture Research Station in Baton Rouge, Louisiana, maintained within a 1200-l recirculating system at a salinity of 10 ppt prepared with sea salt (Crystal Sea, Marinemix Enterprises International Inc., Baltimore, MD), and fed with pelleted catfish feed (Aquaxcel Cargill™, 40% protein) twice daily. The photoperiod was maintained at 12 h light:12 h dark at 27–28 °C. Water was tested every other day by the use of a water-quality kit (Model FF-2, HACH Company, Loveland, CO). The water quality for the systems was maintained as: dissolved oxygen > 6.5 mg/l; total ammonia-N < 3.0 mg/l, nitrite-N < 0.1 mg/l. The guidelines from the Institutional Animal Care and Use Committees of Louisiana State University Agricultural Center were followed in this study.

### 2.2. Sperm collection

Fish were anesthetized with tricaine methanesulfonate (MS-222, Western Chemical Inc., Ferndale, WA) at a dose of 100 mg/l in system water and testes were dissected and weighed after the standard length and body weight were measured. Following dissection, testes were blotted dry with a paper towel, and sperm were mechanically homogenized in a 1.5-ml Eppendorf tube. Sperm samples taken directly (without dilution) from each testis ("dry" sperm) were observed by dark-field microscopy at 200× magnification (Optiphot 2, Nikon Inc., Garden City, NY, USA) to ensure that urine contamination did not activate the samples. Gonadosomatic index (GSI) was calculated as the percentage of testis weight divided by the body weight, and body condition factor (BCF) was calculated as the percentage of the body weight (g) divided by the cube of standard body length (cm).

### 2.3. Solutions used for activating sperm motility

Deionized water (7 mOsmol/kg) and three solutions were used in this study for activating sperm motility: 1) Hanks' balanced salt solution (HBSS). Osmolalities of HBSS ranging from 100 to 1000 mOsmol/kg were prepared by adjusting the water volume using the recipe for 300 mOsmol/kg HBSS (HBSS300: 0.137 M NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.25 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 4.2 mM $NaHCO_3$, and 5.5 mM glucose, pH 7.2). 2) Calcium-free HBSS ($Ca^{2+}$-free HBSS). Solutions ranging in osmolality from 100 to 1000 mOsmol/kg were prepared by using the recipe for HBSS300 without the addition of $CaCl_2$. 3) Sodium chloride (NaCl) solutions. The osmolalites tested with the three solutions above ranged from 100 to 1000 mOsm/kg with an interval of 100 mOsm/kg. The final osmolality of all solutions was confirmed by the use of a vapor pressure osmometer (model 5520, Wescor Inc., Logan, UT).

### 2.4. Motility estimation

Sperm motility was observed at 200× magnification using a dark-field microscope. The motility was expressed as the percentage of sperm that moved actively in a forward direction; sperm that vibrated in place were not considered to be motile. For each sample, sperm motility was estimated for 4–5 different fields.

### 2.5. Experiment I: sperm motility curves

Dry sperm collected from males held at 10 ppt were suspended in buffers with different osmolalities at a ratio of 1:30 (mg testis:μl solution), and 5 μl of diluted sperm suspension were drawn at 30 s, 2 min, and 10 min after mixing to observe motility. Four replicates were produced using testes from four individuals.

### 2.6. Experiment II: sperm motility activation after fish acclimation at different salinities

For acclimation, five males were moved to salinities of artificial seawater of 0, 5, 20, 35, or 50 ppt in 200-l, cylindrical, fiberglass tanks and held for 30 d. The seawater was prepared by mixing of sea salts with fresh water, and the salinity was measured by use of a portable refractometer (RHS-10ATC, Huake Instrument Co., China). The relationship between salinity and osmolality was as follows:

0 ppt = 0–7 mOsmol/kg (deionized water); 10 ppt = 300 mOsmol/kg; 20 ppt = 600 mOsmol/kg; 30 ppt = 900 mOsmol/kg; 33 ppt (full sea water) = 1000 mOsmol/kg. The tanks were aerated and 20 l of water was exchanged each day. Dry sperm collected from fishes from each salinity level were mixed with the buffers at a ratio of 1:30 (yielding about $1–2\times10^8$ cells/ml), and motility of sperm was observed at 30 s, 2 min, and 10 min after mixing as described above. Four replicates were produced at each acclimation-salinity by using testis from four males.

### 2.7. Data analysis

Data were analyzed using SYSTAT 13 (Systat Software Inc., MI). Treatment effects were tested by using analysis of variance (ANOVA), or repeated-measures ANOVA. The relationships among fish characteristics, and sperm motility were analyzed by SYSTAT correlations. Percentage data were arcsine-square-root transformed for normalization before analysis. The significance level was set at $P<0.050$.

## 3. Results

### 3.1. Biological characteristics

In total, the 26 males used in this study had body lengths of $112.0\pm5.7$ mm (mean ± SD) and body weights of $19.93\pm3.81$ g. The testis weights were $134.0\pm56.0$ mg. The GSI calculated was $0.66\pm0.24$, and BCF was $1.40\pm0.12$. Linear correlation analysis showed that body length was significantly correlated with body weight ($P=0.000$) and testis weight ($P=0.011$), and but not with GSI ($P=1.000$) (Fig. 1). Body weight did not show a significant linear correlation with testis weight ($P=0.057$), and BCF did not show a correlation with GSI ($P=1.\ 000$) or testis weight ($P=1.000$) (Fig. 1).

### 3.2. Sperm motility activation

In Experiment I, the three solutions used for motility activation of sperm collected from males cultured at 10 ppt salinity showed different patterns. First, the deionized distilled water (7 mOsmol/kg) did not activate sperm motility. For HBSS, motility was activated at 30 s after mixing with osmolality values ranging from 100 to 900 mOsmol/kg with peak motility ($80\pm10\%$) observed at an osmolality of 500 mOsmol/kg (Fig. 2). After 2 min, the peak motility significantly decreased to $18\pm9\%$ ($P=0.000$), and after 10 min, motility ceased (0%). With $Ca^{2+}$-free HBSS, sperm motility at 30 s was 1–33% at osmolalities of 100 to 1000 mOsmol/kg, the peak motility ($33\pm32\%$) at 600 mOsmol/kg was significantly lower than that ($80\pm10\%$) with HBSS at the peak osmolality ($P\leq0.001$). At 30 s, no significant differences were found among the different osmolalites ($P=0.071$), nor were they found for motility at 2 min ($P=0.101$), but after 10 min, the motility of sperm at lower osmolalities (100–400 mOsmol/kg) increased, and the motility at higher osmolalities (600–900 mOsmol/kg) decreased (Fig. 2). With NaCl as the activating solution, motilities at 30 s, 2 min, or 20 min ranged from 0 to 8%; significantly lower than the motility activated with HBSS ($P\leq0.005$) at peak osmolalites, but the same as the motility activated with $Ca^{2+}$-free HBSS at peak osmolalites ($P\geq0.185$).

### 3.3. Shifting of the motility activation curve after acclimation at different salinities

*Fundulus grandis* acclimated to salinities of 5, 20, 35, or 50 ppt all survived for 30 d. All fish held at 0 ppt (fresh water) stopped eating after 2 wk and died during the acclimation period.

For the three solutions used for activation of sperm motility, patterns similar to those for 10 ppt acclimation in Experiment I were observed, but the peak motilities were shifted from lower osmolalities to higher osmolalites in direct relation to the increase in the

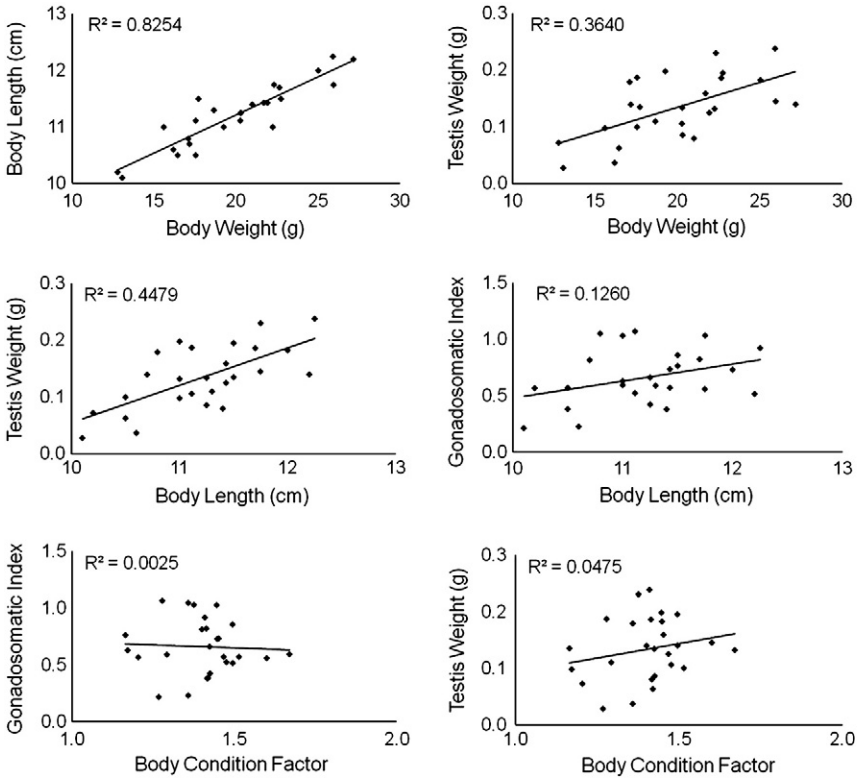


**Fig. 1.** The relationships among body length, body weight, testis weight, gonadosomatic index (GSI, percentage of testis weight to body weight), and body condition factor (percentage of body weight to the cube of body length) of the *Fundulus grandis* males used in this study (N = 26).

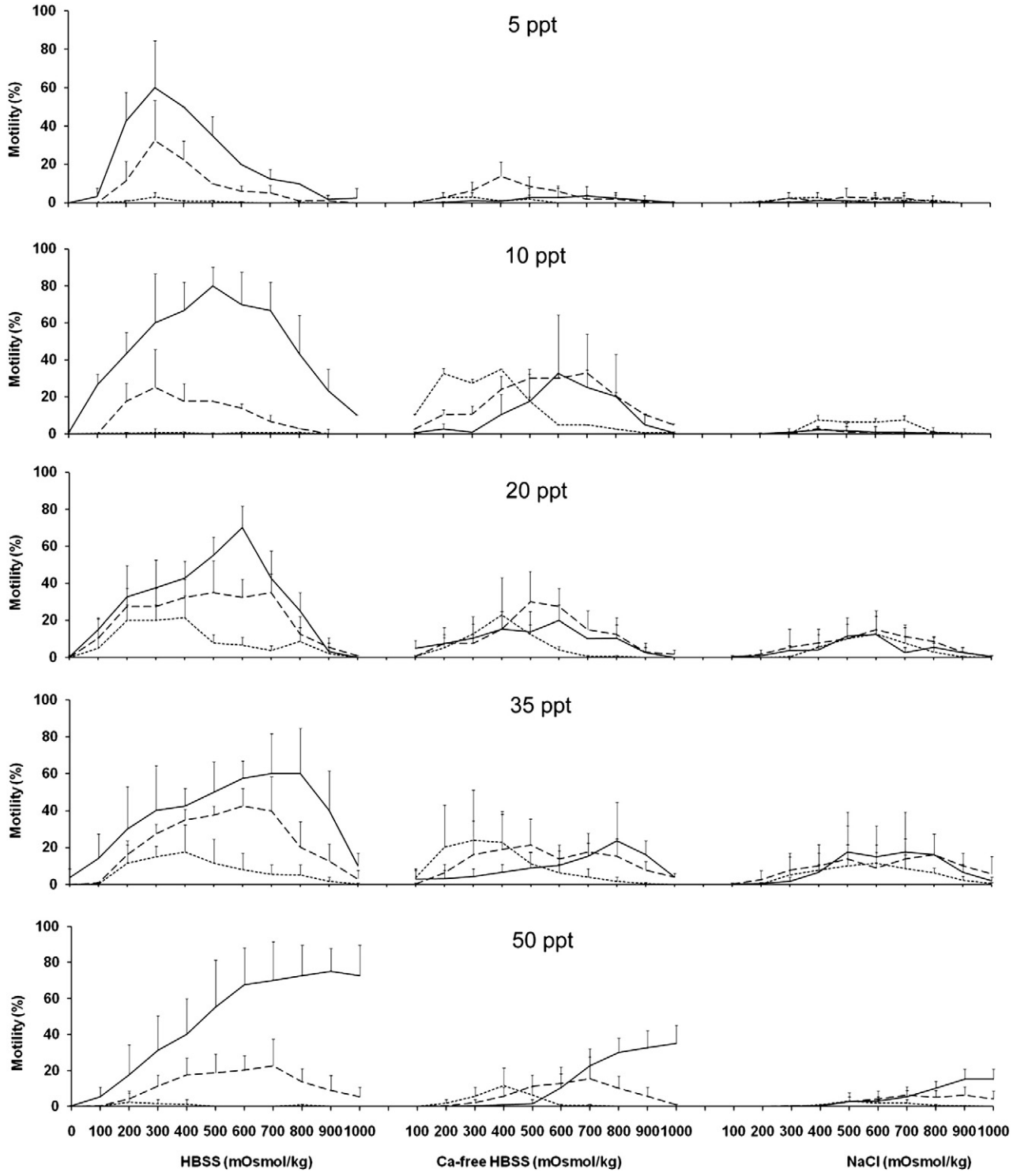


**Fig. 2.** Sperm motility of *Fundulus grandis* activated by mixing with deionized water (7 mOsmol/kg), Hanks' balanced salt solution (HBSS), calcium-free HBSS, or NaCl solutions at osmolalites ranging from 100 to 1000 mOsmol/kg after acclimation for 30 d at salinities of 5 ppt (uppermost panel 1), 10 ppt (panel 2), 20 ppt (panel 3), 35 ppt (panel 4) and 50 ppt (panel 5). Motility estimates were made at 30 s (solid lines), 2 min (dashed lines), and 10 min (dotted lines). Values represented the mean ± SD for individual sperm samples from four fish.

acclimation salinities (Fig. 2). For motility activated by HBSS at 30 s, the osmolalities to activate peak motility were 300 mOsmol/kg (ranging from 200 to 500) for sperm collected from 5 ppt, 500 mOsmol/kg (ranging from 200 to 800) for sperm collected from 10 ppt, 600 mOsmol/kg (ranging from 400 to 700) for sperm collected from 20 ppt, 800 mOsmol/kg (ranging from 200 to 900) for sperm collected from 35 ppt, and 900 mOsmol/kg (ranging from 600 to 1000) for sperm collected from 50 ppt. Thus, 30 d of exposure to different environmental salinities were sufficient to shift the sperm activation profiles in a concentration (salinity)-related fashion.

### 3.4. Function of $Ca^{2+}$ for activating sperm motility

As observed for fishes cultured at 10 ppt, motility activation with $Ca^{2+}$-free HBSS showed a constant pattern for fishes acclimated at different salinities: motility at low osmolalites of $Ca^{2+}$-free HBSS

increased with time at 30 s, 2 min and 10 min, while the sperm motility at higher osmolalites of $Ca^{2+}$-free HBSS decreased with the time (Fig. 2). However, the peak motilities activated with $Ca^{2+}$-free HBSS were significantly lower than those observed when activated with HBSS at each acclimated salinity ($P \leq 0.014$).

## 4. Discussion

### 4.1. A distinct mode for sperm motility activation

Fish sperm are usually quiescent in the testes, and are activated for fertilization after release into the environment. As such, it is not surprising that motility activation of sperm is closely related to the environmental habitats of particular species. For freshwater fishes, sperm motility can typically be activated with hypotonic osmolalities (<300 mOsmol/kg); for marine fishes, sperm motility can typically be activated with hypertonic osmolalities (>300 mOsmol/kg); for viviparous fishes with internal fertilization, sperm motility can be activated with isotonic osmolality (200–400 mOsmol/kg) (Yang et al., 2006). Other mechanisms exist, for example, in salmonid fishes, which are anadromous, motility activation relies on dilution of the potassium concentration in fresh water; for herring *Clupea pallasii* which live in the Pacific ocean and spawn en masse in sheltered bays and estuaries, sperm motility was found to be activated by a protein from the egg chorion which induces calcium influx, sodium efflux, and a membrane depolarization (Vines et al., 2002).

For euryhaline fishes, currently published reports address two species of tilapia, *O. mossambicus* (Linhart et al., 1999; Morita et al., 2003, 2004) and *S. melanotheron* (Legendre et al., 2008), and medaka (Yang and Tiersch, 2009b). *Oreochromis mossambicus* and *S. melanotheron* are widely recognized salinity-tolerant species ranging from fresh water to hypersaline water as high as 120 ppt (Popper and Lichatowich, 1975; Trewavas, 1982). Medaka is considered to be a euryhaline fish, but mostly live in fresh water and can adjust to brackish or sea water by gradual acclimation (Inoue and Takei, 2002). The sperm motility activation of *O. mossambicus*, *S. melanotheron* and medaka from fresh water showed similar patterns, with a wide range of osmolalities including fresh water, hypotonic, and hypertonic solutions (500 mOsmol/kg for tilapia, and 750 mOsmol/kg for medaka) capable of activating sperm motility, but upon activation, sperm motility duration was different, 30 min for sperm of tilapias, and 1 wk for sperm of medaka. For tilapias from sea water, sperm motility could be activated by osmolalities ranging from 0 to 1022 mOsmol/kg (Legendre et al., 2008; Linhart et al., 1999; Morita et al., 2004), and in the presence of 10 mM $Ca^{2+}$, motility could be activated with osmolalites across the 0–1400 mOsmol/kg range (Morita et al., 2004). In contrast to these patterns, sperm motility activation of *F. grandis* in this study showed that osmolalities from 100 to 1000 mOsmol/kg including hypotonic (but not deionized water at 7 mOsmol/kg), isotonic, and hypertonic, could activate sperm motility. Even from these few studies it appears that motility activation in different euryhaline fish species can have different patterns which may be based on their external environments.

### 4.2. Shift of the activation curve by acclimation at different environmental salinities

Another finding in this study was the range of osmolality that activated sperm motility could be shifted higher and broadened as the acclimation salinity increased. This indicated that environmental salinity can alter sperm physiology. A similar phenomenon has been observed in *S. melanotheron* (Legendre et al., 2008) in which sperm collected from fishes acclimated in fresh water, sea water (35 ppt) and hypersaline water (70 ppt) showed activation peaks at 1–350 mOsmol/kg, 300–700 mOsmol/kg, and 450–1200 mOsmol/kg. Also, in *O. mossambicus*, sperm collected from fishes in fresh water and sea water showed activation peaks at different osmolality ranges (0–333 mOsmol/kg vs. 0–1022 mOsmol/kg) (Linhart et al., 1999; Morita et al., 2003, 2004). These observations suggest that a shifting of the response of sperm to environmental salinity may be a characteristic of euryhaline fishes. This does not appear to be the case for the hundreds of stenohaline fresh water and marine species that have been previously studied. Therefore, attention should be accorded to the environment from which euryhaline fishes are maintained or collected. This has been neglected in the vast majority of previous studies of fish sperm. The plasticity of sperm behavior in response to environmental salinity has implications for the purposes of sperm motility activation and use of sperm samples for artificial reproduction. Also, it could provide a valuable tool to tailor the physiology of sperm to the requirements of cryopreservation by manipulation of the salinity of holding tanks, because one of the major challenges sperm encounter during cryopreservation is osmotic shock (Mazur, 1984, 2004). This is also perhaps why cryopreservation of sperm from marine fishes can generally yield higher viability (post-thaw motility and fertility) compared to freshwater fishes (Suquet et al., 2000).

### 4.3. Function of $Ca^{2+}$ in activation of sperm motility

Particular ions play important roles in regulating gamete activation. For fish sperm, the function of ions on motility activation has been reviewed in several reports by different authors (Alavi and Cosson, 2006; Cosson, 2004; Morisawa and Suzuki, 1980; Morisawa et al., 1983). In this study, the results indicated that $Ca^{2+}$ was involved in motility activation because the motility observed in the absence of $Ca^{2+}$ (i. e. $Ca^{2+}$-free HBSS and NaCl solution) was significantly lower than that with $Ca^{2+}$ (regular HBSS with a 1.3 mM $Ca^{2+}$). For tilapias, with the addition of $Ca^{2+}$, the osmolality ranges that activated motility were extended (Legendre et al., 2008; Linhart et al., 1999; Morita et al., 2003, 2004) but osmolality was still a major factor in regulating sperm motility. For example, for *O. mossambicus*, NaCl, KCl and a non-electrolyte (mannitol) initiated motility levels almost equivalent to that observed in the presence of $Ca^{2+}$ at lower osmolalites (less than 100 mOsmol/kg for freshwater acclimated tilapia) (Morita et al., 2003, 2004). In the present study, the function of $Ca^{2+}$ for regulating sperm motility in *F. grandis* was limited to the absence and presence of $Ca^{2+}$ in HBSS. More investigation is needed to define the function of $Ca^{2+}$, such as in relation to concentration. Such research will also be necessary for sperm cryopreservation in which suitable buffers (extenders) need to be developed (Tiersch, 2011).

The sperm characteristics found in *F. grandis* in this research are related to their reproduction mode and habitats, and provide a basis for hypotheses that address the evolution of gametes from estuarine or euryhaline species. *Fundulus grandis* has the ability to inhabit a wide range of salinity from 2 to 70 ppt (Nordlie and Haney, 1998), and this plasticity suggests an explanation for the distinctive sperm characteristics described in this study. *Fundulus grandis* could be unique in this regard, or potentially be representative of euryhaline species. These species, especially small-bodied species, have not received much study of sperm characteristics (Tiersch, 2001), and further research (Yang and Tiersch, 2009a) is warranted based on these and other observations made for medaka (Yang and Tiersch, 2009b). Furthermore, the sperm characteristics found in this study seem more related to those of internal fertilizing fishes than to those of externally fertilizing species, and provide a basis for hypotheses that address whether estuarine or euryhaline species have traits that could have served as pre-adaptations or initiating steps to the processes that led to the evolution of internal fertilization in freshwater fishes.

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