Changes in sperm motility in response to osmolality/Ca$^{2+}$ in three Indonesian fresh water teleosts: Goby (Oxyeleotris marmorata), Java carp (Puntius javanicus), and catfish (Clarias batrachus)

Masaya Morita$^{a,c,*}$, Makoto Okuno$^b$, Endang Sri Susilo$^c$, Bambang Pramono Setyo$^d$, Diptarina Martarini$^d$, Lilik Harnadi$^{a,c}$, Akihiro Takemura$^e$

$^a$ Department of Chemistry, Biology and Marine Sciences, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan
$^b$ Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Meguro-ku, Tokyo 153-8902, Japan
$^c$ Department of Marine Sciences, Faculty of Fisheries and Marine Sciences Diponegoro University, Indonesia
$^d$ Fresh water Fish Hatchery Center, Ngrajeg, Magelang-Fisheries and Marine Sciences, Diponegoro University, Indonesia
$^e$ Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, 3422 Sesoko, Motobu, Okinawa 907-0227, Japan

Received 27 May 2005; received in revised form 12 December 2005; accepted 18 December 2005
Available online 3 February 2006

Abstract

Sperm of most fresh water teleosts become motile when released into the hypotonic fresh water environment, but the role of osmolality and Ca$^{2+}$ on sperm motility is not clear. Osmotic pressure and Ca$^{2+}$ concentrations increase from fresh water to brackish water. Java carp Puntius javanicus and catfish Clarias batrachus live and reproduce only in fresh water. On the other hand, goby Oxyeleotris marmorata can acclimate and reproduce from fresh water to brackish water. In the present study, sperm motility and trajectory were compared among these three Indonesian endemic species. Sperm of Java carp, goby, and catfish begun to move in the hypotonic condition (>200 mOsm/kg). However, the response to Ca$^{2+}$ was different among these teleosts. In the presence of Ca$^{2+}$, Java carp sperm swam in circular paths and immediately become quiescent, suggesting that Java carp sperm motility is activated in hypotonic aquatic environment without Ca$^{2+}$. Goby sperm swam straightforward in the presence or absence of Ca$^{2+}$. Percentages of motile sperm increased in 100–200 mOsm/kg but suppressed by removal of Ca$^{2+}$. Regarding sperm motility and trajectory, no response was found in catfish sperm. These results suggest that a response to Ca$^{2+}$ is different among sperm of the three species and suited to their habitat.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Sperm motility; Ca$^{2+}$; Osmolality; Flagella; Teleost; Goby; Catfish; Carp

1. Introduction

Sperm motility is necessary for efficient fertilization of eggs. In teleosts, regulatory mechanisms of sperm motility are known to be diverse, even though their flagellar axoneme conserves "9+2 structure". Most teleost sperm are quiescent in the testes, because the osmolality of the testicular fluid is isotonic. Sperm motility is initiated when they are released to extracorporeal environment such as hypotonic fresh water or hypertonic seawater. In fresh water teleost sperm, flagellar motility is initiated by the hypoosmotic shock, whereas in marine teleost flagellar motility is initiated by hyper-osmotic shock (Morisawa and Suzuki, 1980). Furthermore, in medaka (Inoue and Takei, 2002, 2003; Kawaguchi et al., in press) and tilapia (Oreochromis mossambicus) (Linhart et al., 1999; Morita et al., 2003, 2004), motility regulatory mechanisms of sperm flagella are modulated to suit the spawning environment when they are in fresh water or acclimated to seawater. In herring sperm, motility initiation required trypsin inhibitor like sperm-activating peptide from the eggs (HSAPS), and the sperm exhibited chemotaxis when they are close to eggs (Yanagimachi and Kanoh, 1953; Yanagimachi, 1957; Yanagimachi et al., 1992; Griffin et al., 1996; Oda et al., 1998). Although regulation of sperm motility is diverse as described above, it
AgFe

An aquatic environment contains several ions such as Na⁺ and Ca²⁺ and these ionic concentrations increase as osmolality increases from fresh water to seawater. It is likely that osmolality and ion composition affect sperm motility regulatory mechanisms. In euryhaline tilapia, sperm motility of fresh water-acclimated tilapia and those of seawater-acclimated tilapia are different (Morita et al., 2003, 2004). Studies on euryhaline tilapia have suggested that sperm of fresh water-acclimated tilapia do not require extracellular Ca²⁺ but sperm of seawater-acclimated tilapia require extracellular Ca²⁺. These reports also imply that a response to extracellular Ca²⁺ is different between teleost fishes, which live in low and high osmolality. One clue for the modulation of sperm flagellar motility regulatory mechanisms is Ca²⁺ concentration of spawning ground. Fresh water does not contain large amount of Ca²⁺ compared to seawater. Thus, it is speculated that tilapia creates sperm in response to osmolality and extracellular Ca²⁺ concentration. In addition, mode of Ca²⁺ mobilization via Ca²⁺ channel for motility activation may also be associated. It is possible that other teleost fishes inhabit both in fresh water and brackish water have same features to Ca²⁺ and osmolality.

In this study, we focused on fresh water teleost fishes, which inhabit both in fresh water and brackish water to study the effect of Ca²⁺ on sperm motility. We observed sperm motility in three species of Indonesian fresh water teleosts. The goby (Oxyeleotris marmorata) can acclimate from fresh water to brackish water. In contrast, the Java carp (Puntius javanicus), and the catfish (Clarias batrachus), live only in fresh water. As indicated above, the concentrations of ions, including Ca²⁺ in their habitat increase from fresh water to brackish water. How do sperm of fresh water teleosts acclimate and utilize environmental differences between fresh water to brackish water? We, therefore, focused on the effect of extracellular osmolality and Ca²⁺ concentration on sperm motility of these fishes. In the presence of Ca²⁺, sperm motility of goby increased in the isotonic condition, however in Java carp and catfish similar changes were not observed. It is likely that sperm motility regulatory mechanisms of fresh water teleosts are also suited to their habitat even though their habitat is hypotonic.

2. Materials and methods

2.1. Fish

Three species of freshwater fishes were used: the goby (O. marmorata), the Java carp (P. javanicus), and the catfish (C. batrachus). They were bred in a freshwater hatchery center of Ngrajek located at Magelang city, central Java, Indonesia.

2.2. Sperm collection

Sperm of Java carp were collected by inserting disposable pipette into the sperm duct. Sperm of goby and catfish was not possible to collect by pressing the abdomen. Hence, these fish were anesthetized with 0.1% (v/v) 2-phenoxyethanol, and testes were dissected out from the abdomen. Sperm were collected at close to sperm duct with a fine disposable transfer pipette from the isolated testes. Collected sperm were stored at room temperature and used within 3 h. Sperm motility in the absence of any treatments was not affected over the course of the experiments. All experiments were carried out at room temperature 20–25 °C.

2.3. Motility observation

NaCl and KCl were used as electrolytes and mannitol as a nonelectrolyte. All solutions contained 10 mM Hepes–NaOH buffer, pH 8.0. Five-millimolar CaCl₂ was added to the NaCl solutions to examine the effect of Ca²⁺ on sperm motility. Semen was diluted into 40 μL of solution on a glass slide and sperm movements were recorded using a video recorder (GR-DVL700, Victor, Japan) and a CCD camera (63W1N, MINTRON, Taiwan) mounted on a phase contrast microscope (DAIKO SCIENCE Co. Ltd, Japan). Percentage of motility was counted from the video recordings. Sperm were counted as motile when they exhibited either progressive movement or spontaneous flagellar beatings even when sperm head was attached to the glass slide. Trajectory was traced using OHP sheet from video recordings.

2.4. Statistical analysis

The data were subjected to two-way ANOVA followed by Fisher’s PLSD for multiple-group comparisons of motility within the same osmolality or different osmolalities. Bonferroni’s post-hoc test was used whenever any significant differences were observed between treatments.

3. Results

3.1. Effect of osmolality on sperm motility of goby, Java carp, and catfish

In Java carp and catfish, sperm began to move after dilution in 10 mM Hepes buffer and stopped moving after 22.7±2.5 and 28.5±2.1 s (means±S.D.), respectively. Goby sperm showed motility for more than 20 min.

Sperm of these fishes were diluted with various concentrations of electrolytes (NaCl and KCl) and a nonelectrolyte (mannitol). As shown in Fig. 1, Java carp sperm showed about 80% motility when they were diluted into hypotonic solutions below 100 mOsm/kg. In KCl solution, sperm were motile up to 100 mM (200 mOsm/kg), however, the motility decreased to about 20% at 125 mM (250 mOsm/kg). In NaCl solution, the motility decreased to 60% at 100 mM (200 mOsm/kg) and almost to 0 at 125 mM (250 mOsm/kg). In mannitol solution, the motility decreased to 60% at 150 mM (150 mOsm/kg), 30% at 200 mM (200 mOsm/kg) and almost 0 at 250 mM (250 mOsm/kg). Effect of NaCl, KCl and mannitol above 150 mOsm/kg on sperm motility was different in Java carp sperm.
In catfish, like as in Java carp (*Puntius javanicus*), sperm was motile under hypotonic conditions (Fig. 2). Approximately 80% sperm showed motility from 10 to 100 mOsm/kg in NaCl, KCl and mannitol solutions. However, motility gradually decreased at 150 mOsm/kg and was completely suppressed at 250 mOsm/kg solutions. Sperm motility was not significantly different at the same osmotic pressure solutions of electrolytes and a nonelectrolyte. The motility of catfish sperm was longer duration at 75 mOsm/kg than at osmolality below 50 mOsm/kg (Fig. 6B).

Fig. 1. Effect of osmotic pressure of electrolytes and a nonelectrolyte on sperm motility of Java carp (*Puntius javanicus*). Sperm were suspended in 10 mM Hepes–NaOH, pH 8.0, containing different concentrations of electrolytes (NaCl and KCl) and a nonelectrolyte (mannitol). Motility was shown in NaCl (blank bar) and KCl (grey bar) and in mannitol (black bar). Values were means±S.D.; N=103–138 sperm from 3 fish for each point.

In catfish, like as in Java carp sperm, sperm was motile under hypotonic conditions (Fig. 2). Approximately 80% sperm showed motility from 10 to 100 mOsm/kg in NaCl, KCl and mannitol solutions. However, motility gradually decreased at 150 mOsm/kg and was completely suppressed at 250 mOsm/kg solutions. Sperm motility was not significantly different at the same osmotic pressure solutions of electrolytes and a nonelectrolyte. The motility of catfish sperm was longer duration at 75 mOsm/kg than at osmolality below 50 mOsm/kg (Fig. 6B).

Fig. 2. Effect of osmotic pressure of electrolytes and a nonelectrolyte on sperm motility of catfish (*Clarias batrachus*). Sperm were suspended in 10 mM Hepes–NaOH, pH 8.0, containing different concentrations of electrolytes (NaCl and KCl) and a nonelectrolyte (mannitol). Motility was shown in NaCl (blank bar) and KCl (grey bar) and in mannitol (black bar). Values were means±S.D.; N=104–256 sperm from 3 fish for each point.

Fig. 3. Effect of osmotic pressure of electrolytes and a nonelectrolyte on sperm motility of goby (*Oxyeleotris marmorata*). Sperm were suspended in 10 mM Hepes–NaOH, pH 8.0, containing different concentrations of electrolytes (NaCl and KCl) and a nonelectrolyte (mannitol). Motility was shown in NaCl (blank bar) and KCl (grey bar) and in mannitol (black bar). Values were means±S.D.; N=108–137 sperm from 3 fish for each point.

Fig. 4. Effect of extracellular Ca\(^{2+}\) on (A) sperm motility and (B) trajectory of goby (*Oxyeleotris marmorata*). Sperm were suspended in NaCl solution (blank bar), NaCl solution containing 5 mM CaCl\(_2\) (grey bar) or 5 mM EGTA (black bar). There were significant differences between motility in the presence of 5 mM CaCl\(_2\) and that in the absence or chelated of Ca\(^{2+}\), *P*<0.01 Motility in solutions more than 100 mOsm/kg. Values were means±S.D.; N=115–263 sperm from 3 fish for each point.
In goby, about 80% sperm showed motility from 10 to 100 mOsm/kg in mannitol solutions (Fig. 3: black bars), but the motility gradually decreased at 150 mOsm/kg and was completely suppressed at 250 mOsm/kg. In NaCl solutions, sperm motility decreased above 150 mOsm/kg and was almost completely suppressed at 200 mOsm/kg (Fig. 3: blank bars), whereas, in KCl solutions, sperm motility decreased above 50 mOsm/kg and was suppressed at 150 mOsm/kg (Fig. 3: grey bar). In goby sperm, large amount of K⁺ was inhibitory on sperm motility. By contrast, in mannitol, sperm retained motility even at high osmolality. The effect of K⁺ and mannitol on motility in the goby sperm was quite different from those of Java carp sperm.

3.2. Effect of extracellular Ca²⁺ on sperm motility

Extracellular Ca²⁺ concentration increases with increase in extracellular salinity. Fresh water environment contains very little Ca²⁺. Brackish water environment close to isotonic osmolality, contains several millimolar Ca²⁺. In euryhaline tilapia, O. mossambicus, acclimated to fresh water, sperm motility increases with millimolar concentration of extracellular Ca²⁺ (Morita et al., 2003).

Goby sperm motility at 150–200 mOsm/kg increased drastically in the presence of Ca²⁺ (Fig. 4A). In addition, the motility was also observed at 250 and 300 mOsm/kg osmolality. By contrast, when extracellular Ca²⁺ was chelated by EGTA, sperm motility was suppressed at osmolalities greater than 100 mOsm/kg (Fig. 4A: black bar), suggesting that Ca²⁺ increases the motility in goby sperm as observed in tilapia (Morita et al., 2003). Trajectory of swimming sperm showed straightforward even in the presence or absence of Ca²⁺ (Fig. 4B). Motility duration also did not change with or without Ca²⁺ (data not shown).

The sperm motility response to extracellular Ca²⁺ in the Java carp sperm was quite different compare to goby sperm. In the presence of Ca²⁺, the percentage of motile sperm in Java carp decreased at all osmolalities (Fig. 5A). Sperm swim in a circular path and became quiescent in the presence of Ca²⁺ within 12 s (Fig. 5B and C). Sperm flagella bent at proximal region like a cane. By contrast, sperm motility increased when extracellular Ca²⁺ was chelated with EGTA (Fig. 5A) and motility duration was longer than in the presence of Ca²⁺ (Fig. 5B). Trajectory of swimming sperm was also in the straightforward direction (Fig. 5C), suggesting that the extracellular calcium induces an asymmetrical beating of flagellum. In Java carp, extracellular Ca²⁺ did not regulate motility initiation but affects the regulatory mechanism controlling symmetry of the waveform.

In catfish sperm, extracellular Ca²⁺ did not affect sperm motility both in motility activation and waveform as shown in Fig. 6A and C. Sperm motility either in the presence or absence of Ca²⁺ did not change significantly. On the other hand, the duration of sperm motility increased around 100–150 mOsm/kg from that at 0–50 mOsm/kg (Fig. 6B). Motility...
duration was not altered either in the presence or absence of Ca²⁺ (Fig. 6B). Trajectory of motile sperm also did not change, and the sperm swam in a straight path either in the presence or absence of Ca²⁺ (Fig. 6C).

4. Discussion

The periods of motility were different among three species. Sperm of Java carp and catfish stopped flagellar movement within 10 to 90 s after the initiation. On the contrary, that of goby was more than 20 min. In the present study, we did not demonstrate the differences in mechanism. It is likely that ways of energy supplemented for flagellar beating of sperm in these fishes are diverse.

Most of the teleosts sperm are quiescent in the isotonic testicular fluid. Sperm motility is initiated when they are released to outer environment such as hypotonic fresh water or hypertonic seawater (Morisawa and Suzuki, 1980). Motility regulatory mechanisms of fresh water teleosts, therefore, are assumed to suit only hypotonic condition (fresh water). In all of three species, sperm motility was initiated when sperm were suspended to hypotonic solutions (Figs. 1, 2, and 3). However, effects of electrolytes and nonelectrolytes on sperm motility initiation were different among species. Electrolytes are known to affect membrane potential. Membrane potential is hyperpolarized when [K⁺]₀ decreases as a result of K⁺ outward current through K⁺ channel. It is speculated that K⁺ efflux does not occur when large amount of K⁺ is present compared to intracellular region containing about 60.5 mM K⁺ (Balkay et al., 1997; Emri et al., 1998). In Java carp and goby, sperm motility initiation was different among electrolytes and nonelectrolyte solutions (Figs. 1 and 3). In goby, sperm motility was inhibited when more than 50 mM (100 mOsm/kg) KCl was present (Fig. 3). On the other hand, in Java carp, sperm motility initiation in the presence of more than 100 mM (200 mOsm/kg) KCl was higher than motility initiation in a nonelectrolyte, mannitol (Fig. 1). In goby and Java carp sperm, K⁺ current between intracellular region and extracellular region may affect motility initiation. Therefore, it is possible that membrane potential is associated with motility initiation in Java carp sperm and goby sperm. It is also possible that an effect of membrane hyperpolarization induced by K⁺ efflux is positive on motility of goby sperm but negative on motility of Java carp sperm. On the other hand, there was no significant difference between the electrolytes and a nonelectrolyte in catfish sperm (Fig. 2), suggesting that sperm motility of catfish is initiated only by osmotic pressure.

Membrane potential is associated with the regulation of several ion channels. It is reported in common carp sperm that a change in membrane potential is associated with a regulation of Ca²⁺ channel. In common carp sperm, change in the membrane potential opens voltage-gated Ca²⁺ channels (Krasznai et al., 1995, 2000) and as a consequence, an increase in intracellular Ca²⁺ concentration is assumed to initiate sperm flagellar motility (Cosson et al., 1989; Krasznai et al., 2000; Takai and Morisawa, 1995). In goby, sperm motility was inhibited when
Ca$^{2+}$ also affects waveform of flagellar motility. Sperm swim circular when symmetry of flagellar beating increases. In Java carp, sperm swim with circular path and became quiescent quickly in the presence of Ca$^{2+}$ (Fig. 5B and C), whereas sperm swam straightforward when extracellular Ca$^{2+}$ was removed (Fig. 5C). In other species, sperm swam straightforward with or without extracellular Ca$^{2+}$ (Figs. 4B and 6B). It is likely that only in Java carp that asymmetry of flagellar beating increased in the presence of Ca$^{2+}$. Flagellum also bent at the proximal region near the head, referred to as the “cane shape”. In demembranated sea urchin sperm model, asymmetry of flagella beating increases with elevated Ca$^{2+}$ concentrations (Brokaw, 1979). Furthermore, in the presence of high concentrations of Ca$^{2+}$ ($10^{-4}$ M), an increase in asymmetry of flagella leads to “cane shape” quiescent state (Gibbons and Gibbons, 1980). Brokaw and Nagayama (1985) reported that calmodulin modulates the asymmetry of sea urchin sperm flagella beating. Together these studies suggest that asymmetry of flagellar beating is induced by Ca$^{2+}$. It is plausible in Java carp that sperm became quiescent as a result of an increase in Ca$^{2+}$, which induces an increase in the asymmetry of flagella beating.

As described above, membrane potential and Ca$^{2+}$ play an important role in regulation of flagellar motility. However, catfish sperm did not match. In catfish sperm, Ca$^{2+}$ had no effect on sperm motility, but motility duration in 100–150 mM was longer than at concentrations below 100 mM (Fig. 6B). Percentage of motile sperm in 150 mM was lower than 100 mM (Fig. 2), suggesting that catfish sperm have ability to increase fertilization success in high osmolality ($>100$ mM) without Ca$^{2+}$. The response to Ca$^{2+}$ was different between Java carp and catfish. In catfish sperm, Ca$^{2+}$ had no effect on sperm motility but motility duration of catfish increased in higher osmolality (Fig. 6B). On the other hand, in Java carp sperm, Ca$^{2+}$ modulates asymmetry of flagellar beating and they became a quiescent state quickly (Fig. 5B and C). It is likely that Java carp sperm suited to low Ca$^{2+}$ environments but catfish sperm may be suited to higher osmolality environment, which contains higher Ca$^{2+}$ concentrations, compare to Java carp sperm.

In conclusion, sperm motility of three Indonesian teleosts, Java carp, goby, and catfish were initiated when they are released in hypotonic environment (below 200 mM). However, the response against extracellular Ca$^{2+}$ was quite different among these species. Sperm motility features such as percentages of motile sperm and motility duration are likely to be suited to the fresh water to brackish water in aspect of extracellular Ca$^{2+}$ concentrations. Arrows indicates the osmolality that could allow sperm motility of each teleosts.

---

**Fig. 7. Schema of possible spawning ground of Java carp, catfish and goby.** As shown in Figs. 1–6, motility-feasible range was different among three species. Sperm motility features such as percentages of motile sperm and motility duration are likely to be suited to the fresh water to brackish water in aspect of extracellular Ca$^{2+}$ concentrations. Arrows indicates the osmolality that could allow sperm motility of each teleosts.
differences among these three species. The Ca$^{2+}$ response seen here among the three species appear to have evolved to adapt to either fresh water containing low Ca$^{2+}$ content or brackish water containing reliably higher Ca$^{2+}$ (Fig. 7).

Acknowledgement

The authors wish to express their deepest gratitude to staff and students of Department of Marine Sciences, Diponegoro University. Thanks are respectfully due to head of Fisheries and Marine Services Government of Central Java, Prof. Dr. Ir. Slamet Budi Prayitno, MSc and staff of his office for their kind support to carry out experiments in east Java of Indonesia. Thanks are sincerely due to Dr. M.M. Vijayan and Dr. N. Aluru, Department of Biology, University of Waterloo, for critically reading the manuscript. This study is supported in part by a Grant-in-Aid for Scientific research (B) (15405029) to AT and 21st Century COE program of the University of the Ryukyus from the Ministry of Education, Culture, Science and Technology, Japan to MM.

References


