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Theriogenology 61 (2004) 1487–1498

Theriogenology

# Activation of sperm motility in striped bass via a cAMP-independent pathway

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Received 12 March 2003; accepted 26 August 2003

## Abstract

The objective of the present study was to identify the effect of osmolality, ions ( $K^+$ ,  $H^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ ) and cAMP on the initiation of sperm motility in striped bass (*Morone saxatilis*). Striped bass spermatozoa remained motile in solutions isotonic to seminal plasma (350 mOsm/kg) until osmolality reached 600 mOsm/kg.  $K^+$  (0–100 mM) had no effect ( $P > 0.05$ ) on sperm motility, and sperm displayed a high percentage of motility over a wide range of pH (6.0–8.5). Sperm motility could be initiated in  $Ca^{2+}$ -free solutions. In contrast, sperm motility was inhibited ( $P < 0.01$ ) by solutions containing  $\geq 10$  mM  $Ca^{2+}$ , and sperm could not be reactivated by a  $Ca^{2+}$ -free solution. This  $Ca^{2+}$  inhibition was not affected by verapamil, a  $Ca^{2+}$  channel blocker. However, if sperm motility was first initiated in a  $Ca^{2+}$ -free solution, the addition of  $Ca^{2+}$  solutions, up to 80 mM, failed to inhibit sperm motility, suggesting that  $Ca^{2+}$  inhibited the initiation of motility, but had no control of motile spermatozoa.  $Mg^{2+}$  solutions had similar inhibitory effects on sperm motility as  $Ca^{2+}$  solutions. Therefore, initiation of motility in striped bass sperm may be related to voltage-gated channels across the cell's plasma membrane. Membrane permeable cAMP did not initiate motility of quiescent, intact striped bass spermatozoa, and motility of demembrated sperm could be activated in the absence of cAMP.

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**Keywords:** Sperm motility; cAMP; Signal transduction; Striped bass

## 1. Introduction

Fish spermatozoa remain quiescent in gonadal seminal plasma in most species that have external fertilization. They become motile at spawning when expelled into the surrounding

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water. Changes in the ionic and osmotic environment of the sperm cells have been identified as two critical external factors that may be responsible for initiating motility in fish spermatozoa [1]. Sperm motility of rainbow trout (*Oncorhynchus mykiss*) is regulated by  $K^+$  concentration, and a  $K^+$  concentration of 40 mM effectively inhibited motility [2]. In contrast to rainbow trout, many species examined appear to be regulated in part by osmolality. Spermatozoa that are quiescent in solutions isotonic to the seminal plasma become motile when exposed to hypotonic environments in freshwater teleosts [3–5] and hypertonic environments in marine teleosts [6,7]. However, for striped bass (*Morone saxatilis*), an anadromous species, we observed that sperm were activated by hypo-, iso-, and hypertonic (up to 600 mOsm/kg) sodium chloride solutions [8,9].

Numerous studies have shown that second messengers, such as cyclic adenosine monophosphate (cAMP) and  $Ca^{2+}$ , play key roles in sperm motility expression in many animal groups, such as mammals [10–13], sea urchins [14], and salmonid fish [15]. Intracellular cAMP concentration controls the net level of phosphorylation of certain specific proteins, especially protein kinase A (PKA) [16,17], that directly leads to initiation of axoneme movement. Calcium increased cAMP through activation of adenylyl cyclase in the spermatozoa of sea urchins [14] and salmonid fish [15,18]. It has been generally accepted [1,15] that external ionic and/or osmotic changes induce the alternation in intracellular concentration of calcium and/or cAMP, and the inhibition of motility may often be due to the inability of the spermatozoa to produce and/or maintain sufficient levels of cAMP to stimulate PKA. However, it has been reported that cAMP and  $Ca^{2+}$  are not necessary for the hypotonic induced initiation of sperm motility in the common carp (*Cyprinus carpio*), and no inhibitory effect of  $Ca^{2+}$  fluxes at the plasma membrane have been observed with sperm from this species [5].

Striped bass spermatozoa are used to fertilize the eggs of white bass (*Morone chrysops*) to produce hybrid striped bass for the striped bass aquaculture industry. This industry increased almost 10-fold from 1986 to 1995 [19], and is now the fourth largest finfish species by value in the US [20]. Cryopreservation of striped bass spermatozoa can aid the industry by amelioration of problems that striped bass and white bass spawning seasons as well as geographic locations are asynchronous. Banked sperm from striped bass can be shipped and stored on site where white bass females are environmentally conditioned to spawn year-round in hatcheries.

It is critical to determine what controls the activation of striped bass spermatozoa. Since the duration of motility in high quality striped bass spermatozoa ranges only from 30 to 60 s [8], it is critical that sperm are not prematurely activated prior to fertilization or cryopreservation. Because striped bass sperm are activated by isotonic solutions, we currently utilize hypertonic cryomedia solutions prior to freezing. However, such solutions carry great risk associated with severe cellular dehydration during periods of equilibration prior to freezing [9]. Development of isotonic solutions that will not activate striped bass sperm would provide good opportunity, through increased time, to manipulate and improve cryopreservation protocols and post-thaw gamete quality at fertilization.

The plasma membranes of fish spermatozoa can be partially removed by detergents, such as Triton X-100 [5,21,22]. This process is defined as demembration, which allows the axoneme to come directly into contact with the external environment. In the present

study, both fresh and demembrated striped bass spermatozoa were applied to evaluate the effects of osmolality, various ions ( $K^+$ ,  $H^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ ) and dibutyryl cAMP (dbcAMP) on the initiation of sperm motility, in our efforts to develop an isotonic extender that might keep striped bass sperm immotile prior to freezing.

## 2. Materials and methods

### 2.1. Collection of spermatozoa and blood

All reagents, unless otherwise stated, were purchased from Fisher Scientific International Inc. (Atlanta, GA). Three-year-old striped bass males were randomly selected from a population maintained under ambient photothermal conditions of the previously described [8] flow-through, 40,000-l circular tank system at the University of Maryland's Crane Aquaculture Facility. Water temperature ranged from 5 to 30 °C during the year. In the spring, males were moved into a 6400-l circular tank, part of a recirculating water system, and held at  $15 \pm 1$  °C for the remainder of the 5-week study. Each fish was given a cholesterol cellulose implant [23] containing 150 µg of mammalian gonadotropin-releasing hormone (Sigma Chemical Co., St. Louis, MO) inserted into the dorsal lymphatic sinus, as previously described for striped bass [24,25]. Three days after administering the implant, the fish were anesthetized in a 70 mg/l quinaldine bath [26] and urine was removed by applying gentle pressure around the urogenital vent. Semen was expressed directly into 50 ml sterile conical tubes and placed immediately on ice. In each experiment, only striped bass sperm samples ( $n = 5$ ) exhibiting  $\geq 90\%$  motility were used. Blood samples were drawn from the caudal vasculature using heparinized needles (21 gauge) and syringes (3 ml).

### 2.2. Analysis of motility

To estimate the percentage of motile sperm, aliquots of semen (approximate 0.01 µl) were placed into a Makler counting chamber (TS Scientific Inc. Perkasie, PA) and quickly mixed with 10 µl of the treatment activating solution. The loaded Makler chamber was immediately placed under a Zeiss model D-7082 compound microscope (Berlin, Germany) at 400 $\times$ . The activation of each sample was recorded on videotape using a Hitachi Model KP-140 video camera (Tokyo, Japan). The percentage of motile sperm was determined by reviewing videotapes and counting spermatozoa (approximately 200–800 cells), then dividing the number of motile sperm by the total number of sperm cells in the field of view. Spermatozoa that simply vibrated or did not show progressive forward movement were not considered in the estimates of motility, as recommended by Billard and Cosson [27]. To test the effect of  $K^+$ ,  $Mg^{2+}$ , or dbcAMP (Sigma Chemical Co.), fresh sperm were activated by the solution containing  $K^+$ ,  $Mg^{2+}$  (0–100 mM) or 0.1 mM dbcAMP. To test the effect of pH, fresh sperm were activated by NaCl solution (100 mOsm/kg) with various pH values from 4.0 to 9.0, adjusted with 1N HCl. To test the effect of  $Ca^{2+}$  on the motility of intact sperm, two methods were used. (i) The semen was diluted 50-fold in a  $Ca^{2+}$ -free solution (240 mM NaCl, 10 mM KCl, 3 mM  $NaHCO_3$ , 0.5 mM EDTA, pH 7.6)

and centrifuged at  $800 \times g$  for 5 min. The precipitate was suspended in the same volume of  $\text{Ca}^{2+}$ -free solution, and 1  $\mu\text{l}$  of this suspension was mixed with 10  $\mu\text{l}$  activating solution containing various concentrations of  $\text{Ca}^{2+}$  (0–100 mM). (ii) The semen was diluted 50-fold in a calcium solution (240 mM NaCl, 10 mM KCl, 1 mM  $\text{CaCl}_2$ , 3 mM  $\text{NaHCO}_3$ , pH 7.6) with or without 100  $\mu\text{M}$  of the calcium channel blocker verapamil (Sigma Chemical Co.) and incubated for 30 min, then the suspension was mixed with activating solution containing the same concentration of verapamil and 20 mM  $\text{Ca}^{2+}$ . Sperm motility was estimated in each test as described above. The duration of sperm motility was timed beginning with the addition of activation solutions to the sperm sample and ending when the majority (approximately 90%) of sperm in the field of view had stopped moving.

### 2.3. Demembration and reactivation of spermatozoa

To partially remove the plasma membranes of the spermatozoa, 10  $\mu\text{l}$  of semen was added to 990  $\mu\text{l}$  of demembration solution containing 240 mM NaCl, 10 mM KCl, 0.5 mM EDTA, 5 mM Tris-HCl, and 0.04% (w/v) Triton X-100, and having a pH of 7.6. After 30 s, 1  $\mu\text{l}$  of the demembrated spermatozoa were placed into the Makler counting chamber and quickly mixed either with 10  $\mu\text{l}$  reactivating solution containing 0.5 mM EDTA, 5 mM Tris-HCl and 1 mM Mg-ATP (Sigma Chemical Co.) as well as various concentrations of NaCl and/or KCl (pH of 7.6) or with the same reactivating solution but without Mg-ATP. To verify that the plasma membranes of spermatozoa were partially broken by the demembration solution, propidium iodide (12  $\mu\text{M}$ ), a fluorescent dye, was used. Spermatozoa stained red with propidium iodide indicated that plasma membranes were not intact. Stained samples were analyzed with a Zeiss Model Axioplan 2 imaging epifluorescent microscope (Berlin, Germany) at  $400\times$ .

### 2.4. Chemical characterization of plasma

Semen and blood samples were centrifuged at  $8000 \times g$  for 30 min, using a Heraeus Model 400-R centrifuge (Hanau, Germany) at  $4^\circ\text{C}$ . Plasmas were decanted, placed in 1.5 ml snap-cap vials, and kept on ice for 30 min before analyses were performed. Osmolality and pH were measured using a Wescor Model 5400 vapor pressure osmometer (Logan, UT) and a Hach Model Sension 2 pH electrode (Loveland, CO), respectively. Sodium, potassium, and calcium were measured using a Perkin-Elmer Model 5100 PC atomic absorption spectrophotometer (Shelton, CT).

### 2.5. Statistical analysis

Statistical analysis was performed with SAS version 8.0 (SAS Institute, Inc., Cary, NC) [28]. Data are presented as means  $\pm$  standard error of the mean (S.E.M.). The assumptions of normality and homogeneity of variances were verified before the parametric tests. Data were analyzed by one-way analysis of variance (ANOVA), and pairwise contrasts were used to identify between means (5% level).

### 3. Results

#### 3.1. Effect of osmolality on intact or demembrated spermatozoa

##### 3.1.1. Intact spermatozoa

Striped bass spermatozoa were fully motile ( $\geq 90\%$ ) immediately after dilution in NaCl or KCl solutions with an osmolality between 40 and 300 mOsm/kg (Fig. 1a). Increasing the osmolality  $>300$  mOsm/kg decreased the percentage of motile sperm, although approximately 60% spermatozoa were motile in solutions isotonic to striped bass seminal plasma (350 mOsm/kg). Sperm motility was not completely inhibited until osmolality reached 600 mOsm/kg. After measurement of the compositions of  $\text{Na}^+$  and  $\text{K}^+$  in seminal plasma of striped bass (Table 1), a solution containing both NaCl and KCl at a comparable 2:1 ratio of  $\text{Na}^+:\text{K}^+$  was tested. This solution exhibited the same effect on striped bass sperm motility as the NaCl or KCl solutions alone.

##### 3.1.2. Demembrated spermatozoa

Fluorescent staining using propidium iodide confirmed that striped bass spermatozoa were demembrated by Triton X-100 (data not shown). In the absence of ATP, demembrated spermatozoa could not be activated. When ATP was added to the reactivating solution, demembrated spermatozoa motility showed the same trend as intact spermatozoa when exposed to increasing osmolality (Fig. 1b). However, the duration of movement lasted only 15–30 s, compared with 30–60 s in intact spermatozoa.

#### 3.2. Effect of $\text{H}^+$ , $\text{Ca}^{2+}$ , $\text{Mg}^{2+}$ on sperm motility

The percentage of motile striped bass sperm was high ( $\geq 90\%$ ) over a broad range of pH values (Fig. 2). Sperm motility decreased ( $P < 0.05$ ) when pH was  $<6.0$  or  $>8.5$ . At a pH of 4.5, only 20% of sperm were motile and those moved primarily in small circular trajectories, compared with primarily linear and to a lesser degree, large circular trajectories when the pH was between 6.0 and 8.5. Additionally at a pH of 4.5, sperm movement decreased rapidly and all cells were immotile after 15 s.

The  $\text{Ca}^{2+}$  concentration of seminal plasma in striped bass was very low ( $0.5 \pm 0.2$  mM), compared to its concentration in blood plasma ( $2.8 \pm 0.1$  mM; Table 1), and seminal plasma in other species such as rainbow trout ( $2.6 \pm 0.19$  mM) [4], and common carp ( $2.0 \pm 0.18$  mM) [2]. Sperm motility was significantly inhibited with 10 mM or higher concentration of  $\text{Ca}^{2+}$  ion in the activating solution (Fig. 3). Only 20–30% of sperm were

Table 1

Ion concentration (means  $\pm$  S.E.M.) in striped bass seminal and blood plasma

Plasma	$\text{Na}^+$ (mM)	$\text{K}^+$ (mM)	$\text{Ca}^{2+}$ (mM)	pH
Seminal plasma	$115 \pm 7.0^a$	$56.1 \pm 5.6^a$	$0.5 \pm 0.2^a$	$7.6 \pm 0.04^a$
Blood plasma	$158 \pm 2.3^b$	$2.7 \pm 0.1^b$	$2.8 \pm 0.1^b$	$7.6 \pm 0.05^a$

Within a column, values with different superscripts (a and b) are different ( $P < 0.05$ ).

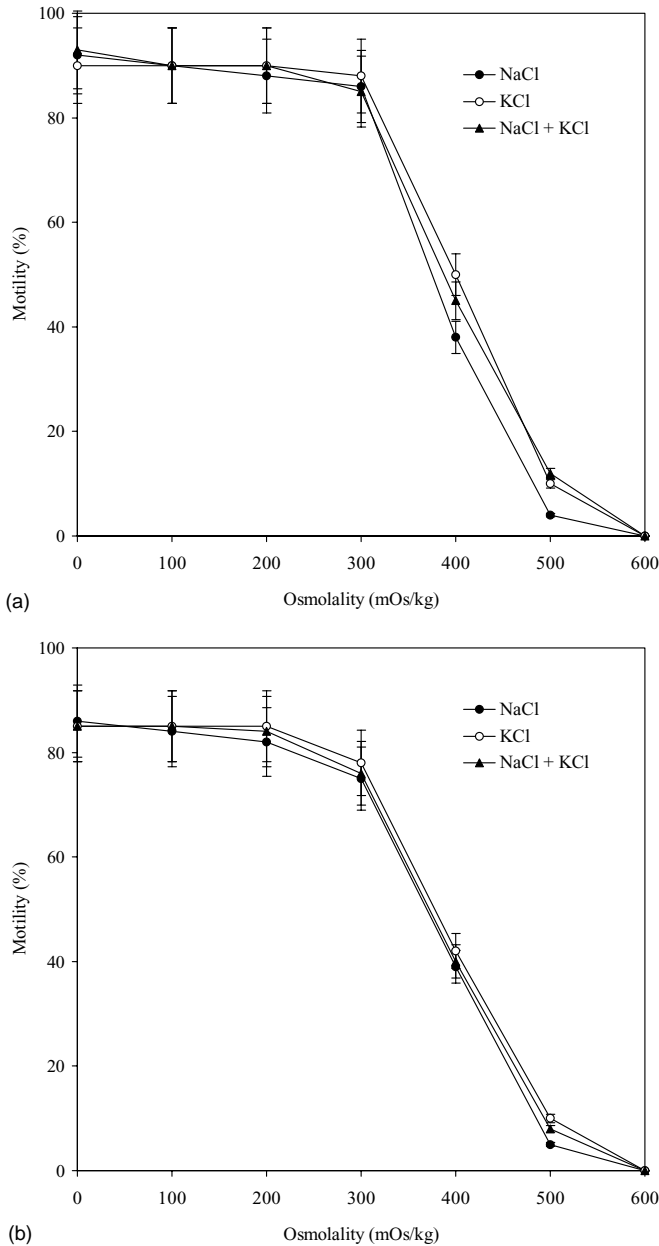


Fig. 1. (a) The percentage of motile sperm (means  $\pm$  S.E.M.) as a function of osmolality and composition of the activating solution. Activating solution contained NaCl, KCl, and the mixture of NaCl and KCl with a ratio of 2:1 ( $\text{Na}^+:\text{K}^+$ ), that mimicked the ratio of  $\text{Na}^+:\text{K}^+$  in seminal plasma. (b) The percentage of motile demembrated sperm (means  $\pm$  S.E.M.) as a function of osmolality and composition of the activating solution. Activating solution contained NaCl, KCl, and the mixture of NaCl and KCl with a ratio of 2:1 ( $\text{Na}^+:\text{K}^+$ ), that mimicked the ratio of  $\text{Na}^+:\text{K}^+$  in seminal plasma.

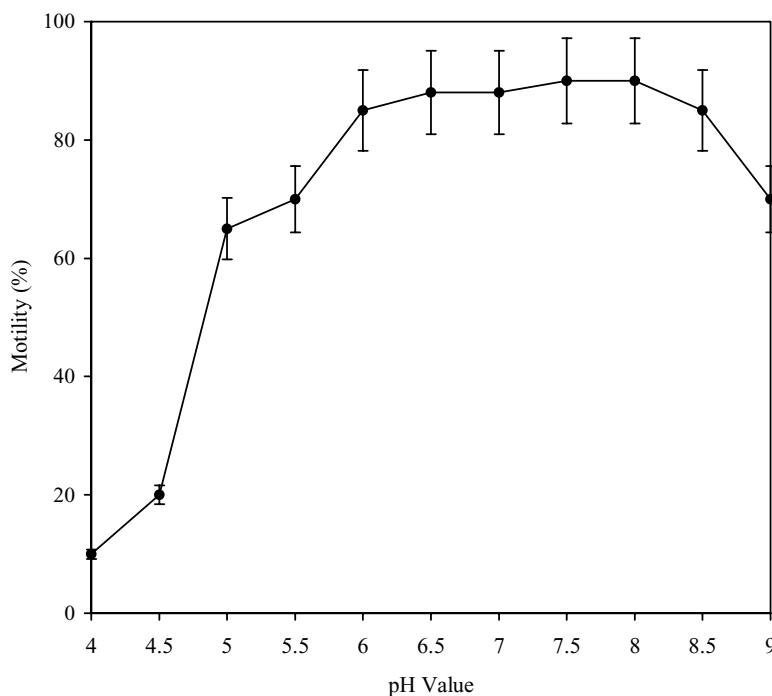


Fig. 2. The percentage of motile sperm (means  $\pm$  S.E.M.) as a function of extracellular pH. Sperm were activated by distilled water.

motile at a concentration of 40 mM  $\text{Ca}^{2+}$ ; furthermore, cells that were moving appeared very slow or weak. The pattern of movement also changed in the presence of a high concentration of extracellular  $\text{Ca}^{2+}$ . Sperm swam in small circular trajectories with very short durations (average, 10–15 s), similar to those described above for low pH. This calcium inhibition of motility was not reversible. Addition of distilled water failed to activate sperm previously diluted in the 40 mM  $\text{Ca}^{2+}$  solution (Fig. 4). However, even the addition of  $\text{Ca}^{2+}$  concentrations as high as 80 mM did not inhibit sperm motility, once sperm were activated by distilled water (Fig. 4). Verapamil, a voltage-dependent calcium channel blocker, had no effect on sperm motility in the presence of 20 mM  $\text{Ca}^{2+}$  (data not shown). High concentrations of  $\text{Mg}^{2+}$  ions also inhibited sperm motility (Fig. 3), and this inhibition could not be overcome by dilution with distilled water.

### 3.3. Effect of dbcAMP on sperm motility

Spermatozoa held under various osmolalities or  $\text{Ca}^{2+}$  concentrations were not affected by the addition of dbcAMP (Table 2). The dbcAMP did not induce the initiation of sperm motility in the NaCl solution with an osmolality of 600 mOsm/kg. No dbcAMP effect ( $P > 0.05$ ) on sperm motility was detected in NaCl solutions with osmolalities of 350 or 500 mOsm/kg, or in the 40 mM  $\text{Ca}^{2+}$  solution.

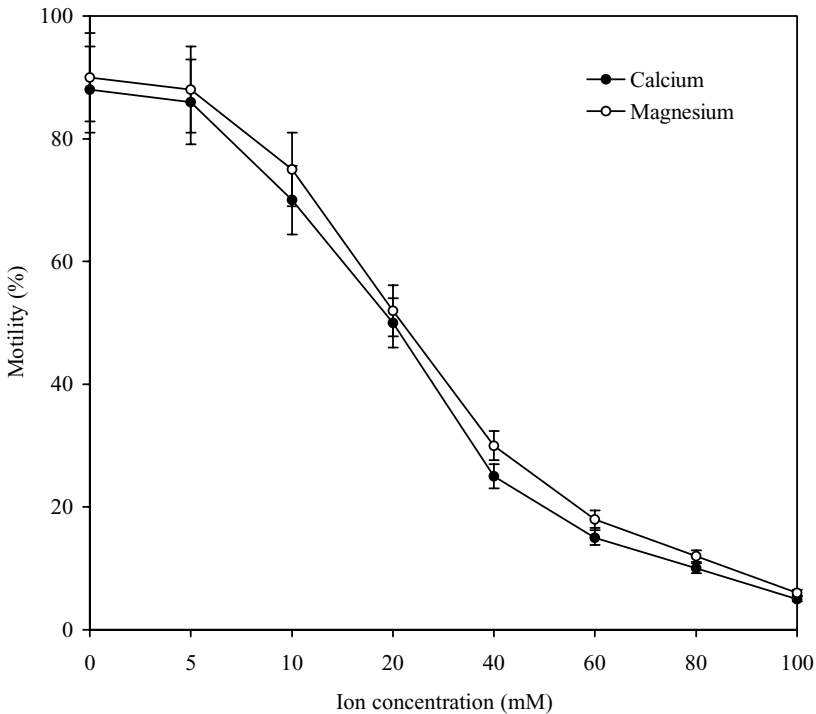


Fig. 3. The percentage of motile sperm (means  $\pm$  S.E.M.) as a function of extracellular  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  concentration. To evaluate the effect of  $\text{Ca}^{2+}$ , sperm suspended in  $\text{Ca}^{2+}$ -free Solution were activated by the activating solution containing various  $\text{Ca}^{2+}$  concentrations. To evaluate the effect of  $\text{Mg}^{2+}$ , sperm were activated by the activating solution containing various  $\text{Mg}^{2+}$  concentrations.

Table 2

Effect of dbcAMP on sperm motility (means  $\pm$  S.E.M.)

	$\text{Na}^+$ (350 mOsm/kg)	$\text{Na}^+$ (500 mOsm/kg)	$\text{Na}^+$ (600 mOsm/kg)	$\text{Ca}^{2+}$ (40 mM)
Control	$56 \pm 3.6^a$	$8 \pm 1.8^a$	0	$25 \pm 2.0^a$
cAMP	$60 \pm 3.1^a$	$9 \pm 1.5^a$	0	$22 \pm 2.3^a$

Fresh semen was activated in solutions with or without (control) 0.1 mM cAMP. Activation solutions included NaCl solution with various osmolalities (350, 500, or 600 mOsm/kg), and  $\text{CaCl}_2$  solution with 40 mM  $\text{Ca}^{2+}$ . Within a column, values with different superscripts (a and b) are different ( $P < 0.05$ ).

#### 4. Discussion

In the present study, motility was triggered in  $>50\%$  spermatozoa by experimental solutions isotonic to seminal plasma, regardless of the  $\text{K}^+$  concentration of those solutions. However, striped bass spermatozoa are immotile in seminal plasma [8]. This suggests that inhibition of motility in striped bass seminal plasma may not be controlled by  $\text{K}^+$  ion concentration, nor by osmolality. The broad range of osmolalities that can

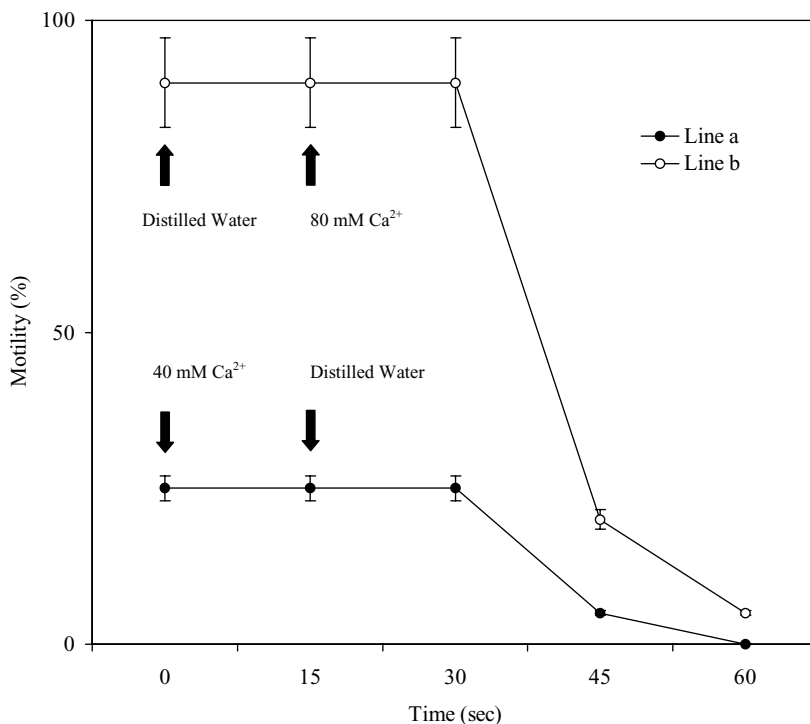


Fig. 4. The relationship between extracellular  $\text{Ca}^{2+}$  concentration and initiation/continuity of sperm motility (means  $\pm$  S.E.M.). In line a, sperm suspended in  $\text{Ca}^{2+}$ -free solution were diluted with 40 mM  $\text{CaCl}_2$  at  $t = 0$  s. At  $t = 15$  s, distilled water was added to the suspension. In line b, sperm suspended in  $\text{Ca}^{2+}$ -free solution were diluted with distilled water at  $t = 0$  s. At  $t = 15$  s, 80 mM  $\text{CaCl}_2$  was added to the suspension.

activate striped bass sperm may be explained by the fact that striped bass is an anadromous species, which migrates from saltwater to freshwater to spawn. To adapt to environmental conditions with such a large variation in osmolality, striped bass sperm may have developed a mechanism by which activation of sperm motility could be initiated in either fresh or brackish waters. A similar effect of osmolality on sperm motility, where sperm remained motile in isotonic solutions (approximately 300 mOsm/kg) was observed in salmonids, and that motility was not completely inhibited until the osmolality reached 400 mOsm/kg [27].

In the present study, demembrated striped bass spermatozoa exhibited the same characteristic patterns of motility as intact spermatozoa, when both were exposed to solutions of various osmolalities. Furthermore, axoneme motility was inhibited in the solution with an osmolality of 600 mOsm/kg. Axoneme motility in demembrated common carp spermatozoa was also resistant to inhibition until osmolality approached 600 mOsm/kg, even though intact spermatozoa remained immotile when osmolality was higher than isotonic values of 250–300 mOsm/kg [5]. It has been reported that high osmolality could directly inhibit axoneme motility in common carp through increased

viscosity of the solution [5]. This suggests that variations in the extracellular osmolality were not directly detected by the common carp spermatozoa axoneme because the plasma membrane acted as a barrier. However, the barrier function of the plasma membrane of striped bass spermatozoa appears to be bypassed, since the axoneme of intact spermatozoa reacted to osmolality the same as that of demembrated spermatozoa.

It has been shown that  $\text{Ca}^{2+}$  is a key factor in sperm motility activation in many species. In trout, when the concentration of free  $\text{Ca}^{2+}$  was reduced below  $10^{-9}$  M (due to addition of EDTA), motility was completely inhibited, but it could be restored by the addition of  $\text{Ca}^{2+}$  [27]. In common carp, the results were less clear. While Perche-Poupard et al. [5] indicated that initiation of motility in common carp spermatozoa was independent of  $\text{Ca}^{2+}$  ions, Krasznai et al. [22] concluded that the  $\text{Ca}^{2+}$  influx from the extracellular environment was the trigger of the initiation of motility in the same species. In the present study, we inferred that the initiation of motility in striped bass spermatozoa does not require the presence of extracellular  $\text{Ca}^{2+}$ . However, since the intracellular  $\text{Ca}^{2+}$  concentration was not monitored, we cannot rule out the potential role of intracellular  $\text{Ca}^{2+}$  for the initiation of sperm motility. Instead of stimulating sperm motility, high concentrations of extracellular  $\text{Ca}^{2+}$  inhibited motility, shortened the duration and modulated the swimming pattern. The sperm of sea bass (*Dicentrarchus labrax*) [29] and bull (*Bos taurus*) [30] had the same swimming pattern of asymmetrical flagellar beating and circular trajectory movement that was directly induced by accumulation of intracellular  $\text{Ca}^{2+}$ . This, together with the observation that diluting the  $\text{Ca}^{2+}$  concentration in the activation solution failed to reactivate striped bass sperm motility, indicated that inhibition may be related to  $\text{Ca}^{2+}$  ion influx into spermatozoa.  $\text{Mg}^{2+}$  concentration also had the same patterns of sperm motility inhibition. Therefore, inhibition may be associated with divalent cations and the associated voltage-gated channels in the plasma membrane. Similar membrane channels may be present in trout spermatozoa. Membrane channels for divalent cations, including  $\text{Ca}^{2+}$  [31],  $\text{Mg}^{2+}$  [32], and  $\text{Sr}^{2+}$  [33] may be present in trout spermatozoa. It was unclear why  $\text{Ca}^{2+}$  concentration in striped bass seminal plasma was so low. We inferred that striped bass spermatozoa may transport extracellular  $\text{Ca}^{2+}$  into the cells to maintain a certain level of intracellular  $\text{Ca}^{2+}$  concentration, which may be important for normal sperm function.

In addition to the  $\text{Ca}^{2+}$ , cAMP has been implicated as an activator of sperm through its protein phosphorylation pathway. Morisawa and Okuno [15] first demonstrated that cAMP was required before ATP can trigger the initiation of sperm motility in rainbow trout. However, present results provided evidence that cAMP did not play a major role in the initiation of sperm motility in striped bass, because the membrane permeable cAMP analog, dbcAMP, was not effective for initiating striped bass sperm motility. Furthermore, demembrated sperm could be reactivated using solution that did not contain dbcAMP.

In conclusion, the mechanism for the initiation of striped bass sperm motility differs from those previously described for other fish such as the rainbow trout and the common carp. Further experimentation is needed to develop isotonic solutions that will not activate striped bass sperm. For example, measuring ionic influx into the sperm cells could provide more information on ionic effects. Future studies to identify plasma membrane channels in striped bass spermatozoa, such as mechanically-activated channels [34], may also provide useful information that may elucidate the mechanism for activation of striped bass spermatozoa.

## Acknowledgements

We thank Dr. William King for his assistance in the critical review of this manuscript. Thanks also go to Daniel Theisen, Chongmin Wang, and Daniel Castranova of our laboratory for providing care and husbandry of the mature striped bass.

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