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Physiological functions of osmolality and calcium ions on the initiation of sperm motility and swimming performance in redside dace, *Clinostomus elongatus*

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ABSTRACT

Reproductive potential of fish stocks is critically dependent on sperm performance in an aquatic environment. The aim of this study is to test hypotheses, which govern the initiation of sperm motility and swimming performance, through physiological functions of osmolality and Ca^{2+} ion, in a threatened species of freshwater fish, the redside dace, *Clinostomus elongatus*. Spermatozoa motility was activated in either ionic or non-ionic media spanning a range of osmolalities. The role of Ca^{2+} channels on induction of spermatozoa motility and velocity was experimentally investigated by diluting sperm in media that contain various Ca^{2+} channel blockers. Results show that initiation of spermatozoa motility is a hypo-osmolality dependent mechanism. Inhibitors for L-type Ca^{2+} channels partially prohibited initiation of spermatozoa motility, while velocity was significantly reduced in both L-type and T-type Ca^{2+} channel blockers. Examination using W-7, an inhibitor for Ca^{2+}-dependent calmodulin, showed significant decreases in spermatozoa motility and velocity. Involvement for Ca^{2+} in axonemal beating was confirmed by significant increases in velocity after adding Ca^{2+} into the activation media, while motility remained unchanged in Ca^{2+} supplemented activation media. Together, these findings suggest the involvement of Ca^{2+} in hypo-osmolality-dependent initiation of spermatozoa motility mediated by activation of Ca^{2+} binding protein in the axoneme of a freshwater fish sperm. Blocking Ca^{2+} exchange through L- or T-type Ca^{2+} channel influences flagellar beating force and leads to decrease in spermatozoa velocity.

1. Introduction

Fish spermatozoa are immotile in the testis or sperm duct prior to spawning (Morisawa et al., 1983a,b). Initiation of spermatozoa occurs after release from the genital papilla into an aquatic environment (Morisawa and Suzuki, 1980). Following initiation of spermatozoa motility, kinetics of motility, including velocity and motility, are key features for fertilization that enable a spermatozoon to locate eggs and subsequently penetrate the micropyle to achieve fertilization (Kudo, 1991; Linhart et al., 2008).

The mechanism of initiation of spermatozoa motility differs among species and is highly dependent on the osmolality of the surrounding media (Alavi and Cosson, 2006; Morisawa, 2008). It is known that a hypo-osmolality and a hyper-osmolality signal is required for triggering initiation of spermatozoa motility in freshwater and marine fish species, respectively (Krasznai et al., 1995, 2000; Takai and Morisawa, 1995; Kho et al., 2003). Following this osmotic signal occurring at the level of the plasma membrane, there are various parameters such as ions and cAMP, which regulate beating of the axoneme (Cosson, 2004; Alavi and Cosson, 2006; Morisawa, 2008). Ions flow across the spermatozoa plasma membrane via specialized ion channels, each of which is distinctly localized in the cell and performs a specific function to regulate the physiological functions of the axonemal molecular structure involved in the initiation of sperm motility (Darszon et al., 2006; Inaba, 2008; Darszon et al., 2011). Within this context, calcium (Ca^{2+}) has been shown to be a major element in triggering sperm motility of fish including freshwater and marine species (Boitano and Omoto, 1992; Tanimoto et al., 1994; Krasznai et al., 2000; Kho et al., 2004; Alavi et al., 2011; Takei et al., 2012). In addition, several Ca^{2+}-dependent proteins have been identified in the axoneme which regulate initiation of spermatozoa motility, flagellar beating patterns, and spermatozoa velocity (Morita et al., 2006; Mizuno et al., 2009).

There are various kinds of Ca^{2+} channels that exhibit species-specific differences in controlling initiation of spermatozoa motility (Darszon et al., 2006). These differences may correspond to the environment of the species and demonstrate both intra- and inter-species

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variations (Darszon et al., 2006, 2011). Particularly, studies have showed that both T-type and L-type Ca^{2+} channel blockers inhibit or suppress initiation of spermatozoa motility, and their role may differ among marine and freshwater species (Kho et al., 2004; Morita et al., 2006). Although, these findings show Ca^{2+}-dependant sperm motility, participation of Ca^{2+} channels needs further investigation to determine whether L-type and/or T-type Ca^{2+} channels are involved in regulation of sperm motility for a particular species.

In the present study, redside dace, *Clinostomus elongates* (J.P. Kirtland), was used as the experimental organism. This is a cyprinid fish native to headwater streams in the basins of the Mississippi River and Lakes Erie, Huron, Michigan, and Ontario (Parker et al., 1988). They typically reside in pools and slow-flowing sections of small streams with clear flowing water, and overhanging flora (McKee and Parker, 1982; Parker et al., 1988). Redside dace live up to four years of age, reaching sexual maturity in their second or third year (Parker et al., 1988; COSEWIC, 2007). They are external fertilizers and exhibit a non-resource-based mating system (Pitcher et al., 2009; Beausoleil et al., 2012). Spawning occurs in gravel riffles between mid-May and early June (Pitcher et al., 2009; Beausoleil et al., 2012). During a spawning event sperm competition is intense, as several males release their sperm (density ranges from 4.4 to 21.4×10^6 sperm/mL, while volume is typically 30.0 ± 20.0 μL) in an attempt to fertilize a clutch of eggs (Beausoleil et al., 2012; unpublished data). Redside dace was once an abundant native species of the fish community in many Great Lake tributaries and played a key ecological role as a bio-indicator of ecosystem health in one of North America's most important freshwater ecosystems (COSEWIC, 2007). However, redside dace populations have declined in many areas across their North American range; for instance, they are listed as Endangered in Canada (COSEWIC, 2007), and in the states of Indiana (IDNR, 2002) and Michigan (Goforth, 2000), and are a species of Special Concern in Wisconsin (Lyons et al., 2000). Thus, pinpointing the precise physiological conditions for "optimal" activation and performance of sperm for this species is warranted.

The objectives of the present study were to investigate how osmolality and ions (K^+, Na^+, and Ca^{2+} ions) impact sperm activation and motility/velocity. Furthermore the role of Ca^{2+} channels on induction of sperm performance was investigated by diluting sperm in activation media that contain various Ca^{2+} ion-channel inhibitors. Results of the present study provide valuable information not only for reproductive biologists, but also for researchers interested in biological conservation programs, and those interested in gene banking for endangered species.

2. Materials and methods

2.1. Chemicals and solutions

Nifedipine, mibefradil dihydrochloride, verapamil hydrochloride, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), sucrose, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium chloride (KCl), and sodium chloride (NaCl) were purchased from ACP (Montreal, QC, Canada), while Tris base, hydrochloric acid (HCl), and calcium chloride ($CaC1_2$) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Fish and gamete collection

Mature males (total length ranged from 7.5 to 8.9 cm) were caught using a 4′ × 10′ × 1/4″ nylon seine (Mid-Lakes Corp., Knoxville, TN, USA) from Rathburn Run in Wayne County, OH, USA (N 40° 48.658′ W 082° 01.400′) and Paint Creek Ohio, USA (N 40° 36.045′ W 082° 03.567′). Fish were typically found in pools between 2 and 5 ft in depth with clear flowing water and overhanging flora. Water temperature, at the time of capture was ~15 °C. Upon capture, redside dace were immediately placed into submerged minnow traps (10″ diameter × 14″ length). Within 5 min fish were removed from the traps and wiped dry. Next, slight pressure was applied on the abdomen of each fish and milt was collected with a micropipette (set at 15 μL) as it was released from the vent (Beausoleil et al., 2012). Extra care was taken to ensure that milt was not contaminated with blood, urine, or feces. Milt samples were then micropipetted into 1000 μL microcentrifuge tubes and held in a cooler filled with frozen ice packs at ~8 °C. Fish were measured (±0.1 cm) and then released.

2.3. Sperm quality assessment

Milt (<0.2 μL) was micropipetted into a chamber of a 2X-CEL glass slide (Hamilton Thorne, MA, USA) and covered with a coverslip (22 × 22 mm). Spermatozoa motility was then activated with 15 μL of activation media (see below) and video-recorded using a CCD black and white video camera (XC-ST50, Sony, Japan) module at 50 Hz vertical frequency, mounted on an external phase contrast microscope (CX41 Olympus, Melville, NY, USA) with a 10× negative-phase magnification objective (Pitcher et al., 2009; Butts et al., 2012). Once recorded, spermatozoa motility traits were analyzed using the HTM-CEROS sperm analysis system (version 12, CEROS, Hamilton Thorne Biosciences, Beverly MA, USA) set at the following parameters: number of frames = 60, minimum contrast = 11, minimum cell size = 3 pixels, and photometer = 55 to 65. To remove the potential effect of drift, spermatozoa which had a VAP (average path velocity) < 20 μm/s and VSL (straight-line velocity) < 10 μm/s were considered to be static and excluded from the analyses (Rudolfsen et al., 2008). Curvilinear velocity (VCL; hereafter referred to as spermatozoa velocity), percent motile spermatozoa, and linearity percentage of the curvilinear track (LIN; calculated as VSL/VCL) were analyzed at various times post-activation (i.e., 10, 15, 20, 30, 40, 50, 60 s). Each video record was manually checked for quality control. Spermatozoa tracks were removed from analyses if the software incorrectly combined crossing tracks of multiple spermatozoa, split the track of a single sperm, or if a spermatozoa swam out of the field of view before adequately being assessed. BSA was not required in the activation medium, as spermatozoa did not stick to the glass slides (see Supplementary material).

2.4. Determination of osmolality and pH

Osmolality was measured using a vapor pressure osmometer (model 5520; Wescor Inc., Logan, UT, USA) and pH was measured using a pH meter (model 5820; VWR Scientific Products, Mississauga, ON, Canada). Mean of the two independent measurements for each constituent was used for further analyses.

2.5. Experiment 1: effects of osmolality on spermatozoa motility kinetics using ionic and non-ionic activation media

Milt was collected and pooled from six mature males in May 2011. To investigate the effect of osmolality, spermatozoa motility traits were evaluated following activation at different osmolalities. Ionic activation media were prepared using KCl and NaCl. KCl activation solutions were tested at 45.0, 67.0, 112.5, 151.0, 197.5, 244.5, 290.5, and 336.0 mOsmol/kg, while NaCl solutions were tested at 51.0, 73.5, 118.5, 151.0, 237.5, 255.5, 321.0, and 365.0 mOsmol/kg. To investigate whether spermatozoa become motile in a non-ionic environment, sucrose solutions were tested at 49.0, 68.0, 106.5, 148.5, 194.5, 243.5, 289.5, and 347.5 mOsmol/kg. All activation solutions were buffered using 20 mM Tris base (26 mOsmol/kg), adjusted to pH 8.0 with HCl and kept refrigerated at ~8.0 °C. In addition, spermatozoa motility was monitored after activation in river water (pH 7.7, 8 mOsmol/kg, ~8.0 °C) and 20 mM Tris base. Three independent

activation trials were conducted per treatment. Mean spermatozoa motility kinetics for each activation trial was used as the experimental replicate.

2.6. Experiment 2: defining the role of Ca^{2+} on spermatozoa motility kinetics

Milt was collected from mature males in May 2012. To study the effects of Ca^{2+} on spermatozoa motility kinetics, different concentrations of $CaC1_2$ (0, 1, and 5 mM) were added to the activation medium (see the Experiment 1: effects of osmolality on spermatozoa motility kinetics using ionic and non-ionic activation media section for further details). This was conducted using 10 males, with each male serving as an experimental replicate. To study the physiological roles of Ca^{2+} on the initiation of spermatozoa motility, Ca^{2+} channel blockers were added to the immobilizing and activation medium. Milt (2 μL) from eight males was added into 1000 μL immobilizing medium (300 mM sucrose, 20 mM Tris pH 8) containing verapamil (10 or 100 μM), mibefradil, (10 or 50 μM), nifedipine (100 μM), or W-7 (100 or 200 μM); note that nifedipine and W-7 were initially dissolved in DMSO, while the other Ca^{2+} channel blockers were dissolved in double distilled water. The spermatozoa suspension was then mixed by agitating tubes for 10 to 15 s and allowed to incubate with each Ca^{2+} channel blocker for 0, 3, and 6 h at 8 °C. After the allotted incubation period initiation of spermatozoa motility was evaluated after activation in 100 mM sucrose, 20 mM Tris, pH 8.0 containing similar concentrations of Ca^{2+} channel blocker (i.e., motility of spermatozoa incubated in 50 μM mibefradil was activated in an activation medium containing 50 μM mibefradil). The final concentration of DMSO (0.05%) in the activation medium had no effect on sperm quality compared to that of the control (see Supplementary material). For the experimental control, spermatozoa were incubated (as above) without a Ca^{2+} channel blocker and then activated with 100 mM sucrose, 20 mM Tris, pH 8, with or without 0.05% DMSO.

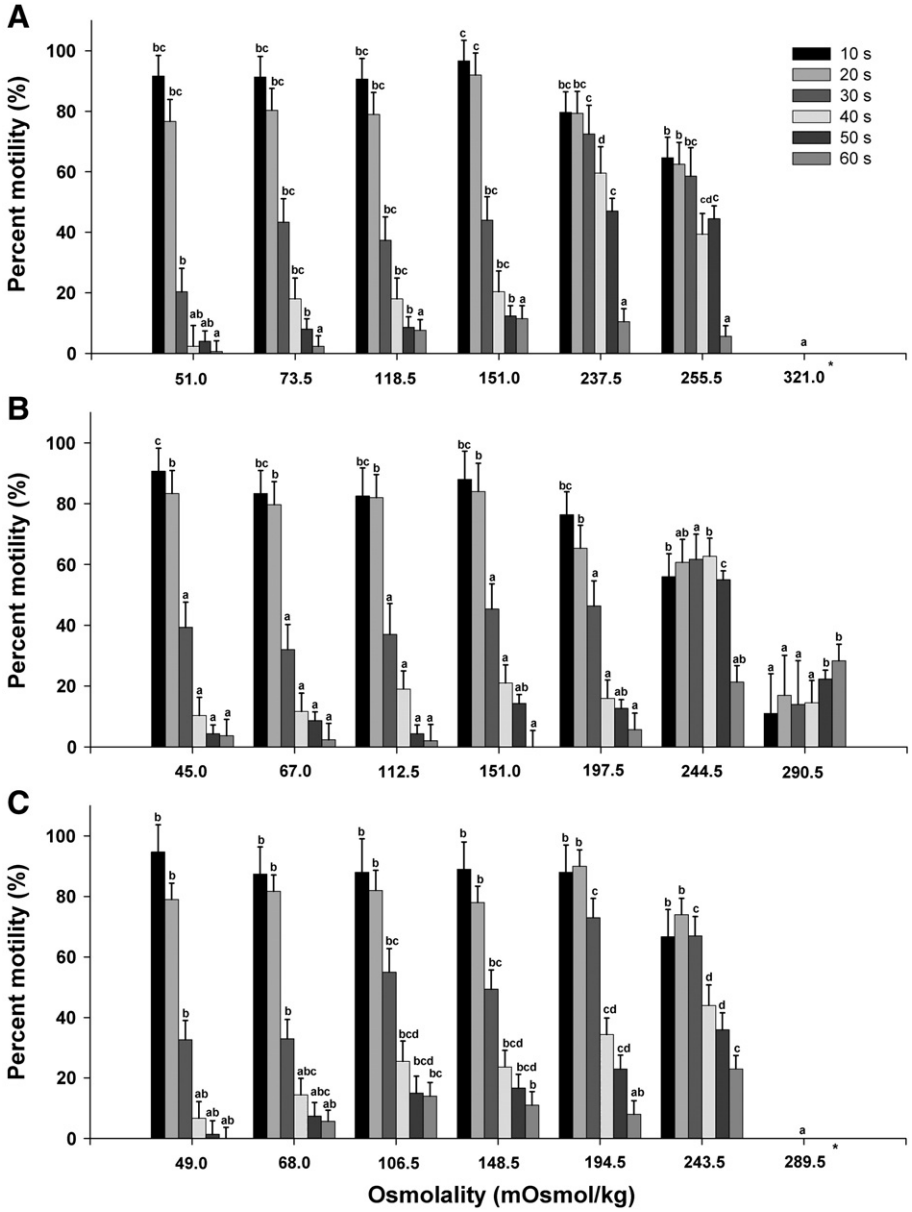

Fig. 1. Effect of osmolality on sperm motility in redside dace, *Clinostomus elongatus*. Sperm from six males were activated with NaCl (A), KCl (B) and sucrose (C) media spanning a range of osmolalities. Within each activation solution, one-way ANOVA models were run at each post-activation time (10 to 60 s); these revised one-way ANOVAs were run because of significant higher-order interactions. Error bars represent least square means standard error. Treatments without a common superscript significantly differed ($P < 0.05$). An asterisk symbol means that there were no motile sperm.

2.7. Statistical analyses

All data were analyzed using SAS statistical analysis software (v. 9.1; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro–Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). Velocity data were $\log_{10}$ transformed to meet assumptions of normality and homoscedasticity when necessary. Percentage data were arcsin square-root transformed. The Satterthwaite and Kenward–Roger procedures were used to approximate denominator degrees of freedom, for all F-tests, for balanced and unbalanced data, respectively (Spilke et al., 2005). Error bars represent least square means standard error.

2.7.1. Experiment 1: effects of osmolality on spermatozoa motility kinetics using ionic and non-ionic activation media

To examine the effect of osmolality on spermatozoa motility for both ionic (NaCl, KCl) and non-ionic (sucrose) activation medium, data were analyzed using repeated measures factorial ANOVA models. Each model contained the dependent variables, osmolality and post-activation time, as well as the accompanying osmolality × post-activation time interaction. In the case of a significant interaction, the model was revised into individual one-way ANOVA models at each post-activation time. These revised models involved only preplanned comparisons and did not include repeated use of the same data, so α-level corrections for a posteriori comparisons were not necessary. Furthermore, to examine the effect of river water and 20 mM Tris base on spermatozoa motility, data were analyzed using repeated measures ANOVA models. Treatment means were contrasted using the Tukey's test.

2.7.2. Experiment 2: defining the roles of Ca^{2+} on spermatozoa motility kinetics

To study the effect of Ca^{2+} on spermatozoa motility, data were analyzed according to the Experiment 1: effects of osmolality on spermatozoa motility kinetics using ionic and non-ionic activation media section. To examine the effect of Ca^{2+} channel blockers on the initiation of spermatozoa motility, data were analyzed using

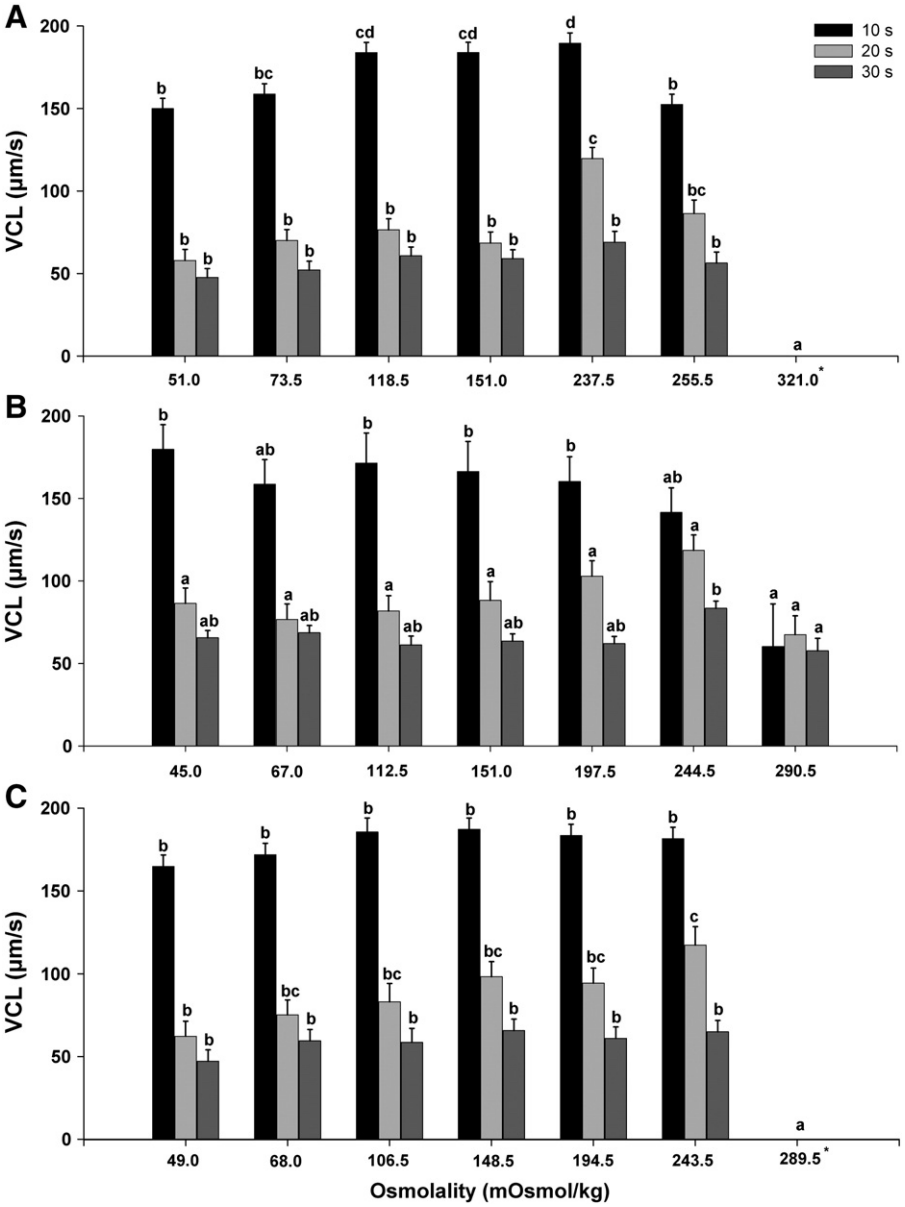

Fig. 2. Effect of osmolality on sperm velocity (curvilinear) in redside dace, *Clinostomus elongatus*. Sperm from six males were activated with NaCl (A), KCl (B) and sucrose (C) media spanning a range of osmolalities. Within each activation solution, one-way ANOVA models were run at each post-activation time (10 to 30 s); these revised one-way ANOVAs were run because of significant higher-order interactions. Error bars represent least square means standard error. Treatments without a common superscript significantly differed ($P < 0.05$). An asterisk symbol means that there were no motile sperm.

repeated measures factorial ANOVA models. Models contained the dependent variables, Ca^{2+} channel blocker and incubation time, as well as the accompanying Ca^{2+} channel blocker × incubation time interaction; models were run separately at each post-activation time. In the case of a significant interaction, the model was revised into individual one-way ANOVA models at each incubation time. If a non-significant interaction was detected the main effects (Ca^{2+} channel blocker and incubation time) were interpreted. To compare each Ca^{2+} channel blocker with that of the control, treatment means were contrasted using the Dunnett's test.

3. Results

3.1. Experiment 1: effects of osmolality on spermatozoa motility kinetics using ionic and non-ionic activation media

For spermatozoa motility, there was an osmolality × post-activation time interaction effect for NaCl, KCl, and sucrose ($P < 0.0001$). Therefore, the models were revised into six separate one-way ANOVAs for times 10, 20, 30, 40, 50, and 60 s (for each ionic/non-ionic activation medium). When the models were revised, osmolality had no effect on spermatozoa motility for NaCl at 60 s (Fig. 1A) and KCl at 30 s (Fig. 1B) ($P > 0.05$). Osmolality had a significant effect on sperm motility for all the other decomposed ANOVA models ($P < 0.01$; Fig. 1).

Both ionic and non-ionic solutions activated spermatozoa motility (Fig. 1). For NaCl, initiation of spermatozoa motility was totally suppressed at 321 mOsmol/kg (Fig. 1A). At 10 and 20 s, the percentage of motile spermatozoa was significantly decreased at 255.5 mOsmol/kg NaCl, while at 30 s a significant decrease in spermatozoa motility was observed at 51.0 mOsmol/kg NaCl (Fig. 1A). At 40 and 50 s, spermatozoa motility was significantly higher when sperm were activated in 237.5 and 255.5 mOsmol/kg NaCl media (Fig. 1A). For KCl, sperm motility was observed at 290.5 mOsmol/kg (ranged from 11.00 to 28.33%; Fig. 1B). At 10 s, the percentage of motile spermatozoa was significantly decreased at 244.5 mOsmol/kg KCl, while at 20 s a significant decrease in motility was observed at 290.5 mOsmol/kg KCl (Fig. 1B). At 40 and 50 s, spermatozoa motility was significantly higher when they were activated in 244.5 mOsmol/kg KCl, while at 60 s motility was significantly higher when sperm were activated in 244.5 and 290.5 mOsmol/kg KCl (Fig. 1B). For sucrose, the initiation of spermatozoa motility was totally suppressed at 289.5 mOsmol/kg (Fig. 1C). At 10 and 20 s, no significant differences in motility were observed between 49.0 and 243.5 mOsmol/kg sucrose (Fig. 1C). Between 30 and 50 s, spermatozoa motility was significantly higher when spermatozoa were activated with 106.5 to 243.5 mOsmol/kg sucrose, while at 60 s motility was significantly higher when spermatozoa were activated in 106.5 and 243.5 mOsmol/kg sucrose (Fig. 1C).

For spermatozoa velocity, there was a significant osmolality × post-activation time interaction effect for NaCl ($P < 0.0001$), KCl ($P = 0.01$), and sucrose ($P < 0.0001$). Therefore, the models were revised into separate one-way ANOVAs for times 10, 20, and 30 s (for each ionic/non-ionic solution). When the models were revised, osmolality had no effect on spermatozoa velocity for KCl at 20 s ($P > 0.05$; Fig. 2B). All other ANOVA models were significant ($P < 0.05$; Fig 2).

For NaCl at 10 s post-activation, spermatozoa velocity followed a dome-shaped function across the osmolality gradient; velocity was highest between 118.5 and 237.5 mOsmol/kg (Fig. 2A). At 20 s, velocity was significantly higher when spermatozoa motility was activated in 237.5 and 255.5 mOsmol/kg NaCl, while at 30 s, no significant difference in spermatozoa velocity was detected between 51.0 and 255.5 mOsmol/kg NaCl (Fig. 2A). For KCl at 10 and 30 s, the percentage of motile sperm significantly decreased at 290.5 mOsmol/kg KCl (Fig. 2B). For sucrose at 10 and 30 s, no significant difference in spermatozoa velocity was detected between 49.0 and 243.5 mOsmol/kg (Fig. 2C).

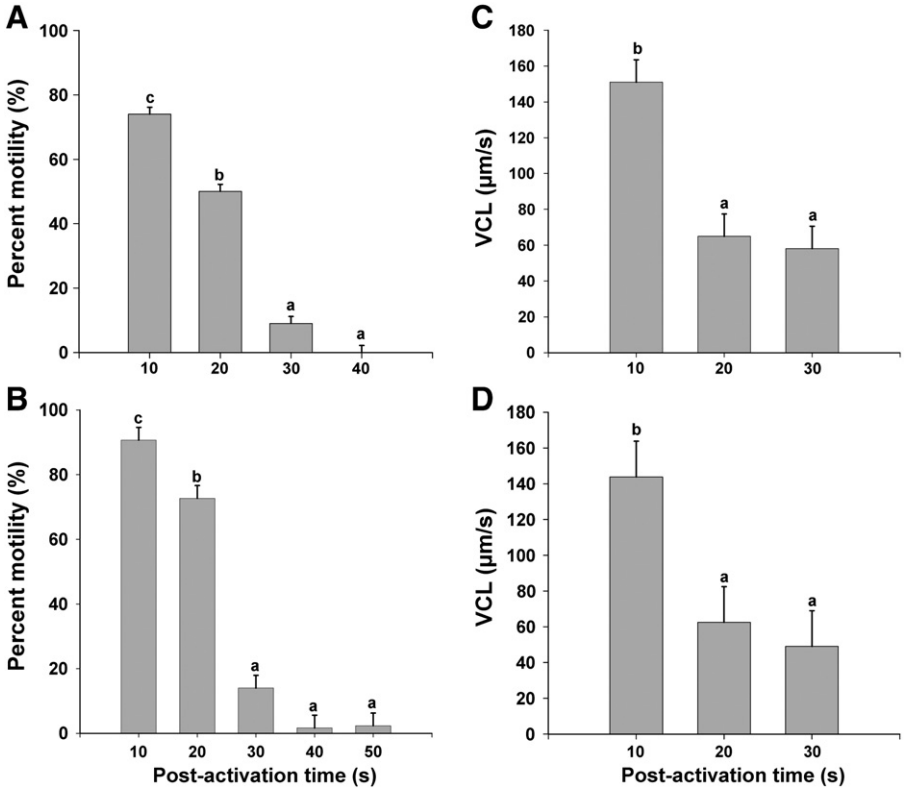

Fig. 3. Effects of river water (pH 7.7, 8 mOsmol/kg, ~8.0 °C) (A, C) and 20 mM Tris base (B, D) on percent sperm motility (10 to 50 s) and velocity (10 to 30 s; curvilinear) in redside dace, *Clinostomus elongatus*. Data were analyzed using repeated measures ANOVA models. Error bars represent least square means standard error. Treatments without a common superscript differed significantly ($P < 0.05$).

When milt was diluted in river water, motility was 74.00 ± 2.17% at 10 s and then significantly decreased to 9.00 ± 2.17% at 30 s ($P < 0.0001$; Fig. 3A). No motile spermatozoa were detected at 40 s (Fig. 3A). When activated in 20 mM Tris, spermatozoa motility was decreased from 90.67 ± 3.94 at 10 s to 2.33 ± 3.94 at 50 s ($P < 0.0001$; Fig. 3B). In river water spermatozoa velocity was decreased from 150.97 ± 12.49 μm/s at 10 s to 58.10 ± 12.49 μm/s at 30 s ($P < 0.01$; Fig. 3C), while in 20 mM Tris velocity decreased from 143.87 ± 19.92 μm/s at 10 s to 49.03 ± 19.92 μm/s at 30 s ($P < 0.001$; Fig. 3D).

3.2. Experiment 2: defining the roles of Ca^{2+} on spermatozoa motility kinetics

For spermatozoa motility, the $CaCl_2$ concentration × post-activation time, and $CaCl_2$ concentration effects were non-significant ($P > 0.05$), while a significant effect was detected for post-activation time ($P < 0.0001$), such that motility decreased at 20 s (Fig. 4A). For spermatozoa velocity, there was a significant $CaCl_2$ concentration × post-activation time interaction effect ($P < 0.05$). Therefore, the models were revised into separate one-way ANOVAs for times 10, 15, and 20 s. When the models were revised, $CaCl_2$ concentration had an effect on sperm velocity at 10 s ($P < 0.001$), such that the swimming velocity of sperm was significantly faster at 1 and 5 mM $CaCl_2$ compared to 0 mM (Fig. 4B). On the contrary, $CaCl_2$ concentration had no effect on spermatozoa velocity at 15 and 20 s ($P > 0.05$; Fig. 4B). There was a significant $CaCl_2$ concentration × post-activation time interaction effect for linearity ($P < 0.05$); therefore the model was revised at each post-activation time. At 10 s, $CaCl_2$ concentration had no effect on linearity ($P > 0.05$; Fig. 5), while at 20 ($P < 0.05$) and 30 s ($P < 0.05$) the $CaCl_2$ effect was significant, such that linearity decreased at higher concentrations (Fig. 5).

For spermatozoa motility the Ca^{2+} channel blocker × incubation time interaction was non-significant at 10, 15, and 20 s ($P > 0.05$); therefore the Ca^{2+} channel blocker and incubation time main effects were interpreted. Incubation time had a significant effect on spermatozoa motility at 10 (Fig. 6A), 15 (Fig. 6B), and 20 s (Fig. 6C), such that motility of sperm decreased overtime ($P < 0.0001$). When sperm were incubated in non-immobilizing media containing 100 μM verapamil and 200 μM W-7, and then activated in media containing Ca^{2+} channel blockers of the same concentration, spermatozoa motility was significantly decreased (to that of the control) at 10 ($P < 0.0001$; Fig. 6D), 15 ($P < 0.001$; Fig. 6E), and 20 s ($P < 0.001$; Fig. 6F).

For spermatozoa velocity the Ca^{2+} channel blocker × incubation time interaction was significant at 10 and 15 s ($P < 0.001$; Fig. 7); therefore, the models were revised to look at the effect of Ca^{2+} channel blocker at each incubation time. When the models were revised at 10 s, Ca^{2+} channel blocker had a significant effect on spermatozoa velocity at 0, 3 and 6 h ($P < 0.0001$), such that spermatozoa velocity was decreased (to that of the control) for 100 μM W-7, and 200 μM W-7 at 0, 3, and 6 h and 50 μM mibefradil, and 100 μM verapamil at 3 and 6 h (Fig. 7A). When the models were revised at 15 s, Ca^{2+} channel blocker had an effect on spermatozoa velocity at 0, 3 and 6 h ($P < 0.0001$; Fig. 7B), such that spermatozoa velocity was decreased (to that of the control) for 50 μM mibefradil at 0 and 3 h, 100 μM nifedipine at 0 h, 100 μM verapamil at 0, 3, and 6 h, 100 μM W-7 at 0 and 3 h, and 200 μM W-7 at 0, 3, and 6 h (Fig. 7B).

The Ca^{2+} channel blocker × incubation time interaction was non-significant for spermatozoa velocity at 20 s ($P > 0.05$), while the incubation time (Fig. 8A) and Ca^{2+} channel blocker (Fig. 8B) main effects were significant ($P < 0.0001$). Data showed that velocity of spermatozoa decreased overtime (Fig. 8A). Furthermore, besides 10 μM mibefradil, all Ca^{2+} channel blockers decreased spermatozoa velocity to that of the control (Fig. 8B).

The Ca^{2+} channel blocker × incubation time interaction was significant for linearity at 10, 15, and 20 s ($P < 0.01$); therefore, the

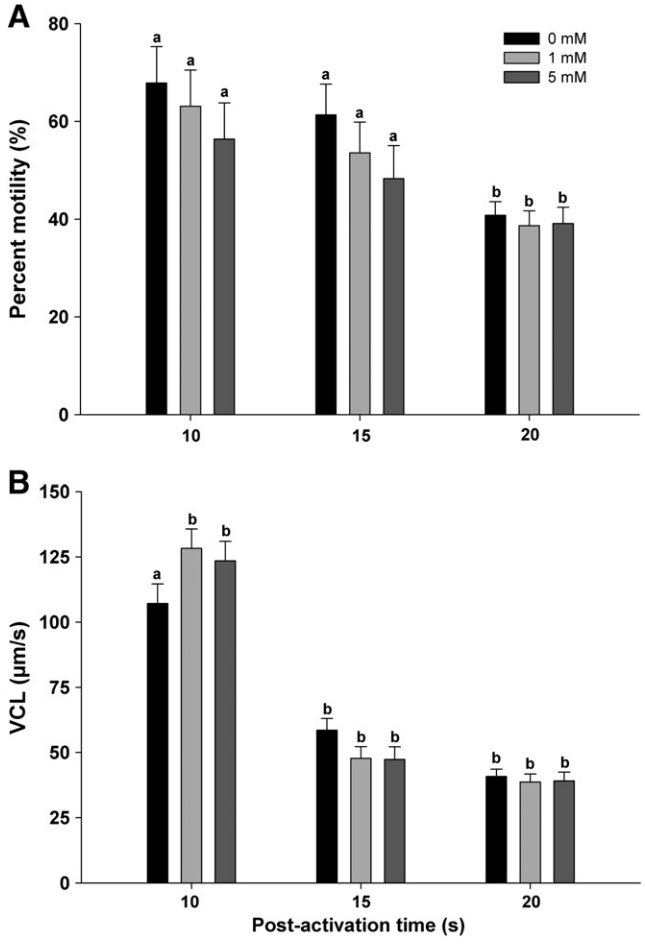

Fig. 4. Effect of calcium (sperm were activated in 0, 1, and 5 mM $CaCl_2$) on percent sperm motility (A) and velocity (curvilinear; B) in redside dace, *Clinostomus elongates* (n = 10). Data were analyzed using repeated measures ANOVA models. For sperm motility, $CaCl_2$ concentration and post-activation time main effects were interpreted due to a non-significant interaction. For percent sperm velocity, one-way ANOVA models were run at each post-activation time (10, 15, and 20 s); these revised one-way ANOVAs were run because of a significant higher-order interaction. Error bars represent least square means standard error. Treatments without a common superscript significantly differed ($P < 0.05$).

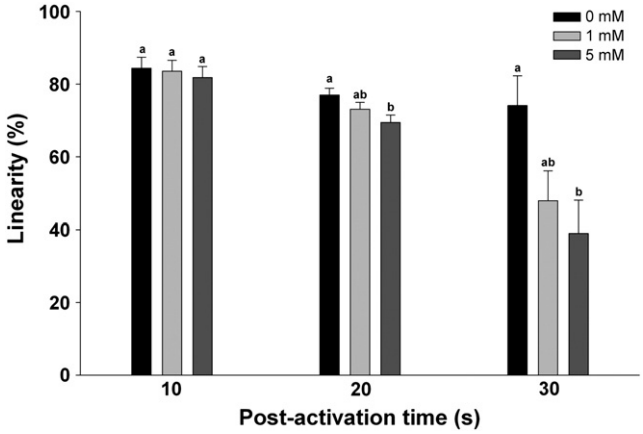

Fig. 5. Effect of calcium (sperm were activated in 0, 1, and 5 mM $CaCl_2$) on linearity of sperm in redside dace, *Clinostomus elongates* (n = 10). Data were analyzed using repeated measures ANOVA models. One-way ANOVA models were run at each post-activation time (10, 15, and 20 s) because of a significant higher-order interaction. Error bars represent least square means standard error. Treatments without a common superscript significantly differed ($P < 0.05$).

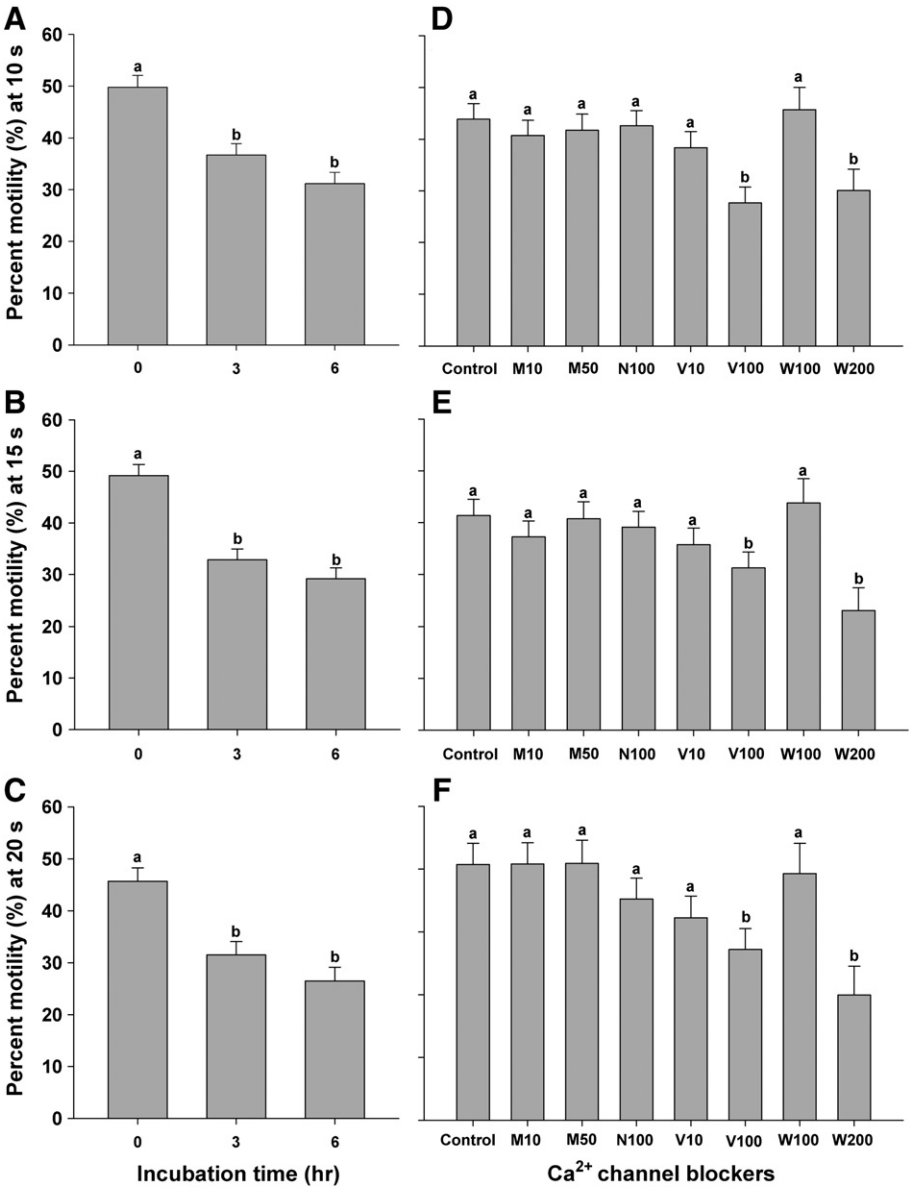

Fig. 6. Effects of Ca^{2+} channel blockers on percent sperm motility in redside dace, *Clinostomus elongates* (n = 8). A non-significant, Ca^{2+} channel blocker × incubation time interaction was detected at 10, 15, and 20 s post-activation. As a result, the incubation time (A, B, C) and Ca^{2+} channel blocker (D, E, F) main effects were interpreted. Error bars represent least square means standard error. Treatment means were contrasted using the Dunnett's test. Treatments without a common superscript significantly differed ($P < 0.05$). M10 = 10 μM mibefradil, M50 = 50 μM mibefradil, N100 = 100 μM nifedipine, V10 = 10 μM verapamil, V100 = 100 μM verapamil, W100 = 100 μM W-7, W200 = 200 μM W-7.

models were revised to look at the effect of Ca^{2+} channel blocker at each incubation time. When the models were revised at 10 s, Ca^{2+} channel blocker had a significant effect on linearity at 0, 3 and 6 h ($P < 0.05$).

4. Discussion

4.1. Hypo-osmolality dependent sperm activation

We found that redside dace sperm were activated in either ionic (NaCl or KCl) or non-ionic (sucrose) activation media, suggesting that ions are not essential to produce hypo-osmotic signal for the initiation of sperm motility in this species. These finding are consistent with other externally fertilizing freshwater fishes, such as common carp, *Cyprinus carpio* (Morisawa et al., 1983b), Eurasian perch, *Perca fluviatilis* (Lahnsteiner et al., 1995), common barbell, *Barbus barbus* (Alavi et al., 2009), Northern pike, *Esox lucius* (Alavi et al., 2009), and vimba bream, *Vimba vimba* (Alavi et al., 2010). Nevertheless, ions may still be involved in stimulation of spermatozoa motility initiation by changing properties of the plasma membrane, including its osmotic potential and its ionic conductance (Cosson et al., 1999; Morisawa et al., 1999).

Osmolality of redside dace seminal plasma is ~280 mOsmol/kg (n = 6 males; unpublished data) and we observed that spermatozoa motility could be activated at osmolalities lower than 300 mOsmol/kg in activation media composed of NaCl, KCl, or sucrose. These results suggest that a hypo-osmolality signal lower than that of seminal plasma triggers spermatozoa motility in this species, which is similar to other freshwater species (Morisawa, 2008). On the contrary, this is different from salmonid and sturgeon species, in which spermatozoa motility is activated at hypo-osmolality condition associated with a decrease in extracellular KCl (Morisawa et al., 1983a; Alavi et al., 2011); because it has been shown that KCl at 1 mM prohibits initiation of spermatozoa motility in these species (Morisawa et al.,

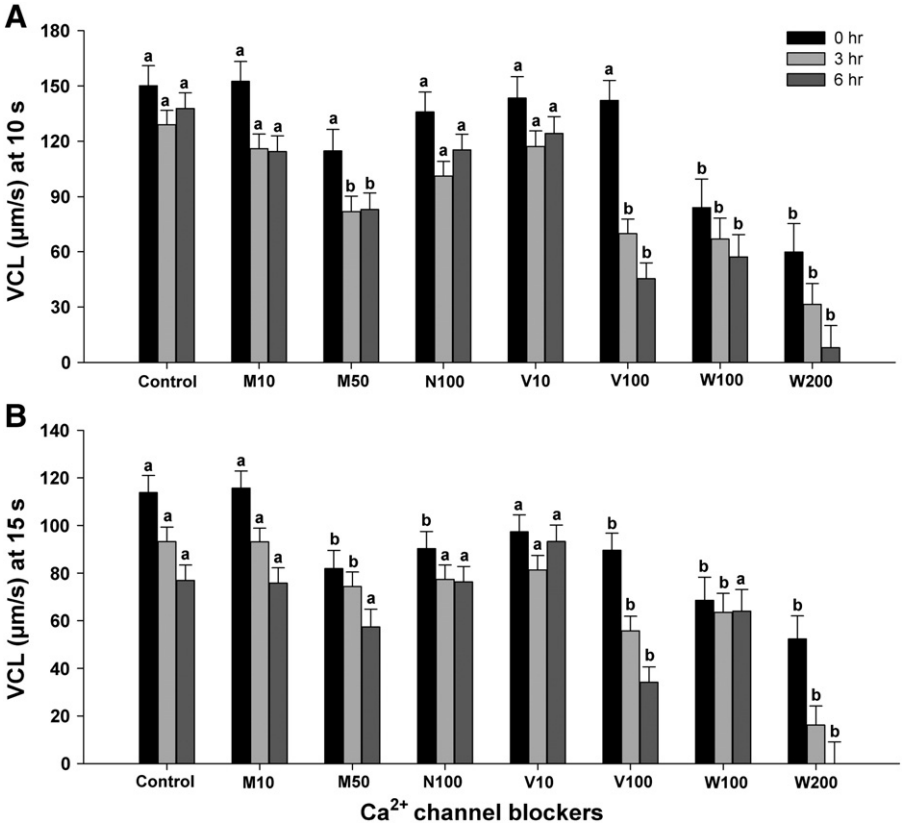

Fig. 7. Effects of Ca^{2+} channel blockers on curvilinear velocity (VCL) at 10 and 15 s post-activation in redside dace, *Clinostomus elongates* (n = 8). One-way ANOVA models were run to determine the effect of Ca^{2+} channel blocker for each incubation time (0, 3, and 6 h); these revised one-way ANOVAs were run because of significant Ca^{2+} channel blocker × incubation time interactions at 10 and 15 s post-activation time. Error bars represent least square means standard error. Treatment means were contrasted using the Dunnett's test. Treatments without a common superscript significantly differed ($P < 0.05$). M10 = 10 μM mibefradil, M50 = 50 μM mibefradil, N100 = 100 μM nifedipine, V10 = 10 μM verapamil, V100 = 100 μM verapamil, W100 = 100 μM W-7, W200 = 200 μM W-7.

1983a; Alavi et al., 2011). Compared to freshwater fishes, marine fish spermatozoa are different because hyper-osmolality condition is required for ionic-independent initiation of spermatozoa motility (Billard et al., 1995; Takai and Morisawa, 1995).

4.2. Effects of osmolality on sperm activation

In addition to involvement of osmolality in triggering spermatozoa motility, several studies showed that osmolality of the activation medium influences spermatozoa motility or velocity (Morisawa and Suzuki, 1980; Morisawa et al., 1983a,b; Billard et al., 1995; Dreanno et al., 1999). Our study is the first that illustrates adaptation of redside dace spermatozoa to extracellular osmotic condition. In general, spermatozoa motility was totally suppressed at 60 s post-activation in hypo-osmolality condition; i.e. river water, river water containing 20 mM Tris or NaCl and KCl (25 mM) and sucrose (25 and 50 mM). At the same time post-activation, sperm motility was measured above 10 to 20% at osmolalities of 105 to 255 mOsmol/kg. Furthermore, spermatozoa motility evaluated at 60 s post-activation was lower after activation in river water compared to spermatozoa activated with NaCl, KCl, and buffered river water. The lower percentage and shorter duration of spermatozoa motility following activation in low hypo-osmolality condition may be due to damage of the flagellum mediated by alternations in the sperm plasma membrane, which appears in forms of cytoplasmic blebs (or irregular bulges) along the flagellum and curling at the base of the flagella; these alternations ultimately impair the propagation of waves and shorten the length of the flagellum (Alavi et al., 2009). Therefore, optimum osmolality for activation of redside dace spermatozoa may be considered between 105 and 225 mOsmol/kg, which is similar to that of Northern pike spermatozoa, in which the highest motility or velocity has been recorded following activation at osmolalities between 125 and 235 mOsmol/kg (Alavi et al., 2009). The optimum conditions for common carp, tilapia, *Oreochromis mossambicus*, and Eurasian perch spermatozoa have been recorded at 90 to 110 (Billard et al., 1995; Cosson, 2010), 100 to 300 (Linhart et al., 1999), and 100 to 150 mOsmol/kg (Alavi et al., 2007), respectively. These inter-species differences may be related to ionic composition and osmolality of the seminal plasma, time of stripping during reproductive season, and environmental factors regulating spermiation (i.e., temperature and photoperiod) (Billard et al., 1995; Alavi et al., 2008).

4.3. Physiological role of Ca^{2+} in the initiation of sperm motility in redside dace

Although initiation of spermatozoa motility in freshwater fish is a hypo-osmolality dependent mechanism, intracellular signaling is still required to transfer the osmotic shock into the motility apparatus called the axoneme. Within this context, two major messengers have been suggested; cAMP and Ca^{2+} (Morisawa, 2008). The potential role of Ca^{2+} was investigated in this study, because it has been shown that sperm activation in fish is a cAMP-independent mechanism (Krasznai et al., 2000). This is similar to marine fish (Oda and Morisawa, 1993; Takai and Morisawa, 1995), but in contrast to sperm of salmonids and sturgeon in which cAMP is required for the initiation of spermatozoa motility (Inaba et al., 1998; Kho et al., 2003; Alavi et al., 2012). Our results showed that initiation of spermatozoa motility decreased when spermatozoa were incubated in the presence of a L-type Ca^{2+} channel blocker (verapamil) or in the presence of an inhibitor for Ca^{2+}/calmodulin activated phosphodiesterase

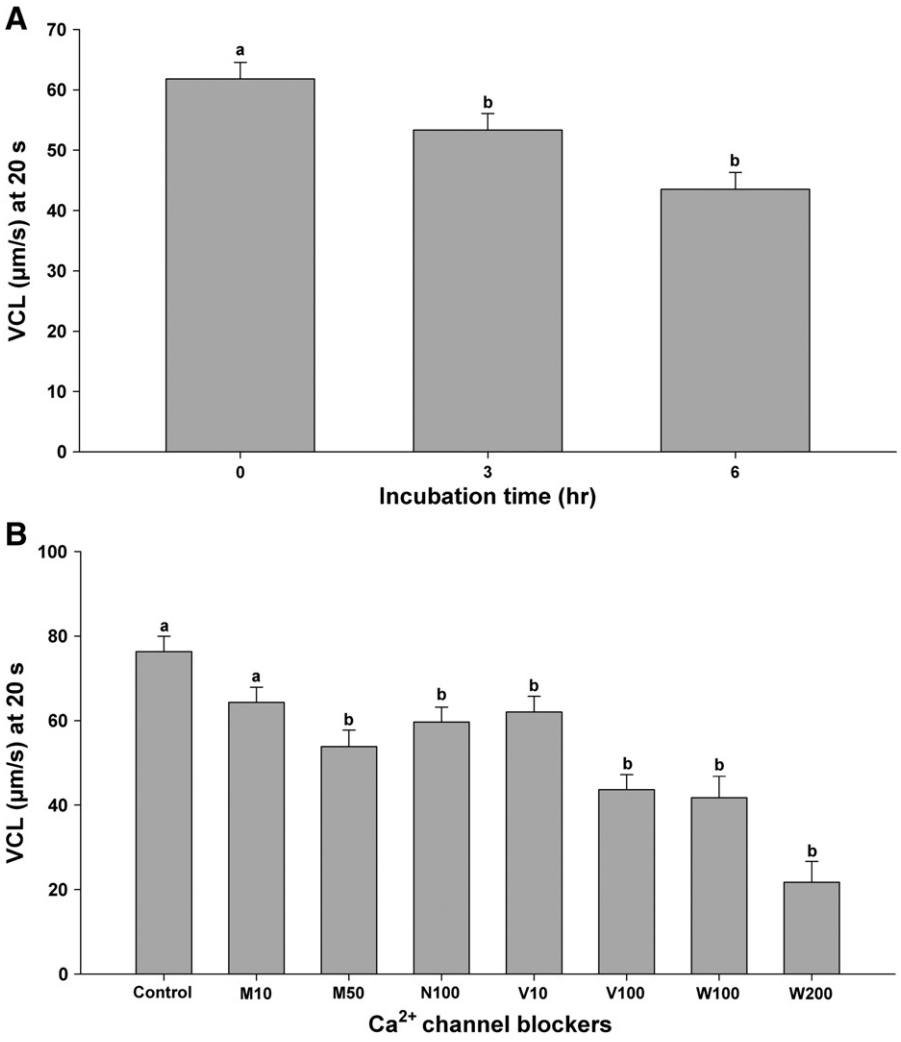

Fig. 8. Effects of Ca^{2+} channel blockers on curvilinear velocity (VCL) at 20 s post-activation in redside dace, *Clinostomus elongates* (n = 8). A non-significant, Ca^{2+} channel blocker × incubation time interaction was detected at 20 s post-activation. As a result, the incubation time (A) and Ca^{2+} channel blocker (B) main effects were interpreted. Error bars represent least square means standard error. Treatment means were contrasted using the Dunnett's test. Treatments without a common superscript significantly differed ($P < 0.05$). M10 = 10 μM mibefradil, M50 = 50 μM mibefradil, N100 = 100 μM nifedipine, V10 = 10 μM verapamil, V100 = 100 μM verapamil, W100 = 100 μM W-7, W200 = 200 μM W-7.

(W-7); and then activated in media containing the same concentrations of these inhibitors. Moreover, spermatozoa velocities were decreased either in the presence of L-type (nifedipine) or T-type (mibefradil) Ca^{2+} channel blockers, as well as in the presence of W-7. Together, these findings are strong evidence that Ca^{2+} plays a physiological role at two different sites. Firstly, at the level of the plasma membrane, as blocking Ca^{2+} channels lead to a decrease in the percentage of motile sperm. Secondly, the molecular structure of the axoneme is another target for Ca^{2+} because incubation of sperm with W-7 leads to a significant decrease in both spermatozoa motility and velocity.

Our study shows involvement of L-type Ca^{2+} channels in activation of redside dace sperm, which is similar to that of sturgeon (Alavi et al., 2011) and different from salmonids, and common carp sperm where both L-type and T-type Ca^{2+} channel blockers completely prohibit sperm activation (Tanimoto and Morisawa, 1988; Krasznai et al., 2000; Kho et al., 2004). It is important to note that none of these studies showed complete suppression of sperm activation in the presence of Ca^{2+} channel blockers and only significant decreases were observed following incubation. Similar to the present study, suppression of spermatozoa motility in the presence of inhibitors for Ca^{2+}/calmodulin activated phosphodiesterase has been previously shown in most species. For instance, it was demonstrated that inhibitor for Ca^{2+}-dependent calmodulin phosphorylation could prohibit sperm activation in common carp (Krasznai et al., 2000), salmonids (Kho et al., 2004), tilapia (Morita et al., 2006), and sturgeon (Alavi et al., 2011). These inter-species differences to various Ca^{2+} channel blockers and similarities to Ca^{2+}/calmodulin activated phosphodiesterase is biologically interesting. Overall, it suggests that active ion channels, involved in the initiation of spermatozoa motility, may differ among species, but the molecular structure of axoneme is well conserved.

4.4. Effects of supplemented extracellular Ca^{2+} on sperm motility kinetics

As mentioned above, Ca^{2+} channels are involved in Ca^{2+}-dependent axonemal beating required for the initiation of spermatozoa motility. In the present study, we added Ca^{2+} in the activation medium to investigate its role on spermatozoa motility and velocity. It was observed that adding 1 or 5 mM Ca^{2+} does not influence initiation of spermatozoa motility but significantly increases spermatozoa velocity. In non-supplemented activation medium with Ca^{2+} (control with 0 mM Ca^{2+}), sperm motility was also activated. Similar effects of Ca^{2+} on spermatozoa velocity have been reported in both freshwater (such as rainbow trout, Eurasian perch, sturgeon) and marine fish, in which Ca^{2+} increases sperm velocity (Oda and Morisawa, 1993; Alavi et al., 2007, 2011; Takei et al., 2012). The observed significant impact of supplemented Ca^{2+} on sperm velocity, in the present study, suggests

potential Ca^{2+} influx upon hypo-osmolality, which in turn regulates phosphorylation of Ca^{2+} binding proteins because inhibitor for Ca^{2+}–calmodulin protein (W-7) prohibited sperm motility and velocity.

The effects of extracellular Ca^{2+} can be also examined by use of EGTA, a chelator agent with a very high affinity to Ca^{2+}. Our future research should address whether extracellular Ca^{2+} is a critical element for the initiation of sperm motility as previous studies on both freshwater and marine fish species revealed that spermatozoa motility could be initiated in EGTA supplemented activation medium (Oda and Morisawa, 1993; Alavi et al., 2011; Takei et al., 2012). In contrast, addition of EGTA can prohibit sperm activation in other freshwater species, such as common carp, chum salmon, and masu salmon (Tanimoto and Morisawa, 1988; Krasznai et al., 2000; Kho et al., 2004; Takei et al., 2012). The literature shows high inter-species differences in response to addition or elimination of extracellular Ca^{2+} by EGTA which may be related to the composition of the seminal plasma, and ionic composition of spawning habitats, which influences the response of sperm to ionic media following release in the aquatic environment (Takei et al., 2012).

5. Conclusions

The present study shows that initiation of redside dace spermatozoa motility is initiated with a hypo-osmolality dependent mechanism. Inhibitors for L-type Ca^{2+} channels could partially prohibit initiation of spermatozoa motility, while spermatozoa velocity was reduced in both L-type and T-type Ca^{2+} channel blockers. This may be evidence for involvement of Ca^{2+} in both initiation of spermatozoa motility and regulation of axonemal beating. Further examination using W-7, an inhibitor for Ca^{2+}-dependent calmodulin, showed significant decreases in both spermatozoa motility and velocity. Involvement of Ca^{2+} in axonemal beating was confirmed by significant increases in spermatozoa velocity observed after adding Ca^{2+} into the activation media. Sperm motility remained unchanged in Ca^{2+} supplemented activation media. Therefore, it is important to perform further experiments using a chelating agent for extracellular Ca^{2+} or using Ca^{2+} ionophore for better understanding of the role of extracellular and intracellular Ca^{2+} stores.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2013.05.011.

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