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Research Article

The role of alkalinization-induced Ca^{2+} influx in sperm motility activation of a viviparous fish Redtail Splitfin (*Xenotoca eiseni*)†

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Abstract

Mechanisms regulating sperm motility activation are generally known in oviparous fishes, but are poorly understood in viviparous species. The mechanism of osmotic-shock induced signaling for oviparous fishes is not suitable for viviparous fishes which activate sperm motility within an isotonic environment. In addition, the presence of sperm bundles in viviparous fishes further complicates study of sperm activation mechanisms. The goal of this study was to establish methodologies to detect intracellular Ca^{2+} signals from sperm cells within bundles, and to investigate the signaling mechanism of sperm activation of viviparous fish using Redtail Splitfin (*Xenotoca eiseni*) as a model. Motility was assessed by classification of bundle dissociation and computer-assisted sperm analysis, and intracellular Ca^{2+} was assessed using the fluorescent probe Fura-2 AM. Bundle dissociation and sperm motility increased with extracellular Ca^{2+} and pH levels. Intracellular Ca^{2+} signals were detected from sperm within bundles, and increased significantly with extracellular Ca^{2+} and pH levels. Major channel blockers known to inhibit Ca^{2+} influx ($NiCl_2$, ruthenium red, $GdCl_3$, SKF-96365, nimodipine, verapamil, methoxyverapamil, mibefradil, NNC 55–0396, ω-Conotoxin MVIIC, bepridil, and 2-APB) failed to inhibit Ca^{2+} influx, except for $CdCl_2$, which partially inhibited the influx. We propose a novel mechanism for motility regulation of fish sperm: an alkaline environment in the female reproductive tract opens Ca^{2+} channels in the sperm plasma membrane without osmotic shock, and the Ca^{2+} influx functions as a second messenger to activate motor proteins controlling flagella movement.

Summary Sentence

Extracellular alkalinization can induce Ca^{2+} influx, which activates sperm within bundles of a viviparous fish.

Key words: Ca^{2+}, ion channels, sperm, sperm motility and transport, signal transduction, fish reproduction.

Introduction

Viviparity has evolved repeatedly and independently among fishes [1], amphibians [2], reptiles [3], and mammals [4]. Viviparity has been proposed to have evolved in fishes in the Devonian period (approximately 380 million years ago) among placoderms [5, 6], and is currently represented in about 40 families of chondrichthyans, 1 monotypic family of coelacanths, and 13 families of teleosts [1]. Internal fertilization is the basis of viviparity, and a shift of sperm activation from external aqueous environments to the female reproductive tracts is an essential step toward evolution of internal fertilization. In applied practice, sperm from viviparous fishes are used in (1) development of germplasm repositories via sperm cryopreservation for imperiled species and biomedical research models [7], (2) artificial insemination [8], and (3) research activities addressing behavioral and evolutionary studies [9]. Knowledge of the cellular and molecular mechanisms controlling sperm motility activation is important for the optimization and standardization of these activities, and understanding the evolution of internal fertilization. Such mechanisms are generally well studied in oviparous fishes, but are poorly understood in viviparous species.

During natural spawning, fish sperm are discharged from the male reproductive tract into the aqueous environment surrounding oviparous fishes, or into the female reproductive tract in viviparous species. Sperm are immotile inside the testes, but the changing physiochemical conditions upon spawning trigger a sequence of intracellular signaling pathways that initiate sperm motility. In oviparous species, changing in the osmotic pressure or ionic concentration around sperm cells usually initiates motility through increases of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via Ca^{2+} influx into the sperm. For example, in the freshwater species Common Carp (*Cyprinus carpio*) and Rainbow Trout (*Oncorhynchus mykiss*), spawning of sperm into hypotonic environments with reduced extracellular K^+ triggers a transient shift of cell membrane potential, which activates voltage-dependent Ca^{2+} channels, resulting in the Ca^{2+}influx that initiates sperm motility [10–12]. In the saltwater species Stone Flounder (*Kareius bicoloratus*), Grass Puffer (*Takifugu niphobles*), and Panther Puffer (*T. pardalis*), Ca^{2+} release from intracellular stores in response to hypertonicity activates sperm motility [13]. However, shifting osmolarity does not seem to be the trigger of motility activation for viviparous fishes, which transfer sperm through isotonic environments (usually approximately 300 mOsmol/kg for freshwater species) from the male to female reproductive tracts. For example, sperm motility of Guppy (*Poecilia reticulata*, a viviparous species of the Family Poeciliidae) is highest in isotonic solution, but is inhibited in hypotonic and hypertonic solutions [14].

It is possible that the mechanisms regulating motility in viviparous fishes are different from those reported for oviparous species. Other than osmotic pressure, the composition and concentration of extracellular ions can affect isotonic activation. Sperm from the Guppy had the highest degree of activation in $CaCl_2$ solution compared to other divalent and monovalent cations, but no increases in intracellular Ca^{2+} signals were observed during extracellular Ca^{2+}-induced activation [15], which suggests a Ca^{2+}-sensing mechanism at the plasma membrane level. Previously, we demonstrated the ability of extracellular Ca^{2+} to activate sperm from Redtail Splitfin (*Xenotoca eiseni*), a freshwater endangered viviparous species belonging to the Goodeidae family [16]. However, the mechanism underlying this effect remains unknown. Goodeidae and Poeciliidae are two freshwater viviparous families within the Cyprinodontiformes with independent evolution of viviparity [17]. For example, poeciliid females can store sperm for months but goodeids are believed only hold sperm for days within the female reproductive tract [16]. As such, it is possible that mechanisms by which ions regulate sperm motility activation among different taxa of viviparous fishes are different. In addition to ions, environmental pH can affect isotonic activation of sperm motility. In mammalian sperm, alkaline pH induces sperm activation by stimulating Ca^{2+} influx through the CatSper Ca^{2+} channel [18, 19]. In fish sperm, this effect has not been reported and it is believed that pH affects sperm motility, but the effects are not as pronounced as osmotic pressure and ions for oviparous species [20–22].

A challenge to study cell signaling mechanisms in viviparous fish is the packing of sperm cells in un-encapsulated (spermatozeugmata) (Figure 1A, the preparation and process of bundle suspension for SEM was as described previously [16]) or encapsulated (spermatophores) bundles [23–25]. The presence of sperm bundles is sporadic among vertebrates and usually accompanied with internal fertilization and viviparity in fishes. The formation of sperm bundles is seen in invertebrates including nematodes [26], annelids [27], arthropods [28], and molluscs [29], and in vertebrates including amphibians [30], chondrichthyans [31], and teleosts [32]. Bundles are believed to facilitate the systematic transfer of sperm from male to female [32], possibly by resisting the flushing effect of water when bundles cannot be delivered directly into the female reproductive tract. Hence, they also represent a challenge for studying intracellular signals. For example, when using flow cytometry cells pass individually through a laser beam to detect fluorescence and optical characteristics of each cell [33], but this is not suitable for sperm bundles. To date, no effective methodologies have been established to investigate intracellular signaling for sperm bundles.

In the present study, we investigated for the first time the signaling mechanism of sperm activation of viviparous fish using Redtail Splitfin as a model. The specific objectives were to establish a methodology to detect intracellular Ca^{2+} signals in sperm within bundles, and to determine the effects of extracellular Ca^{2+} and pH on the Ca^{2+} signaling mechanism controlling sperm motility activation.

Materials and methods

Chemicals

The ratiometric calcium indicator Fura-2 AM was purchased from Cayman Chemical Company (www.caymanchem.com), SKF-96365 and ω-Conotoxin MVIIC were from Alomone Labs (www.alomone.com), and Tricaine methanesulfonate (MS-222) was from Western Chemical, Inc (www.syndel.com). All other chemicals were from Sigma-Aldrich (www.sigma-aldrich.com).

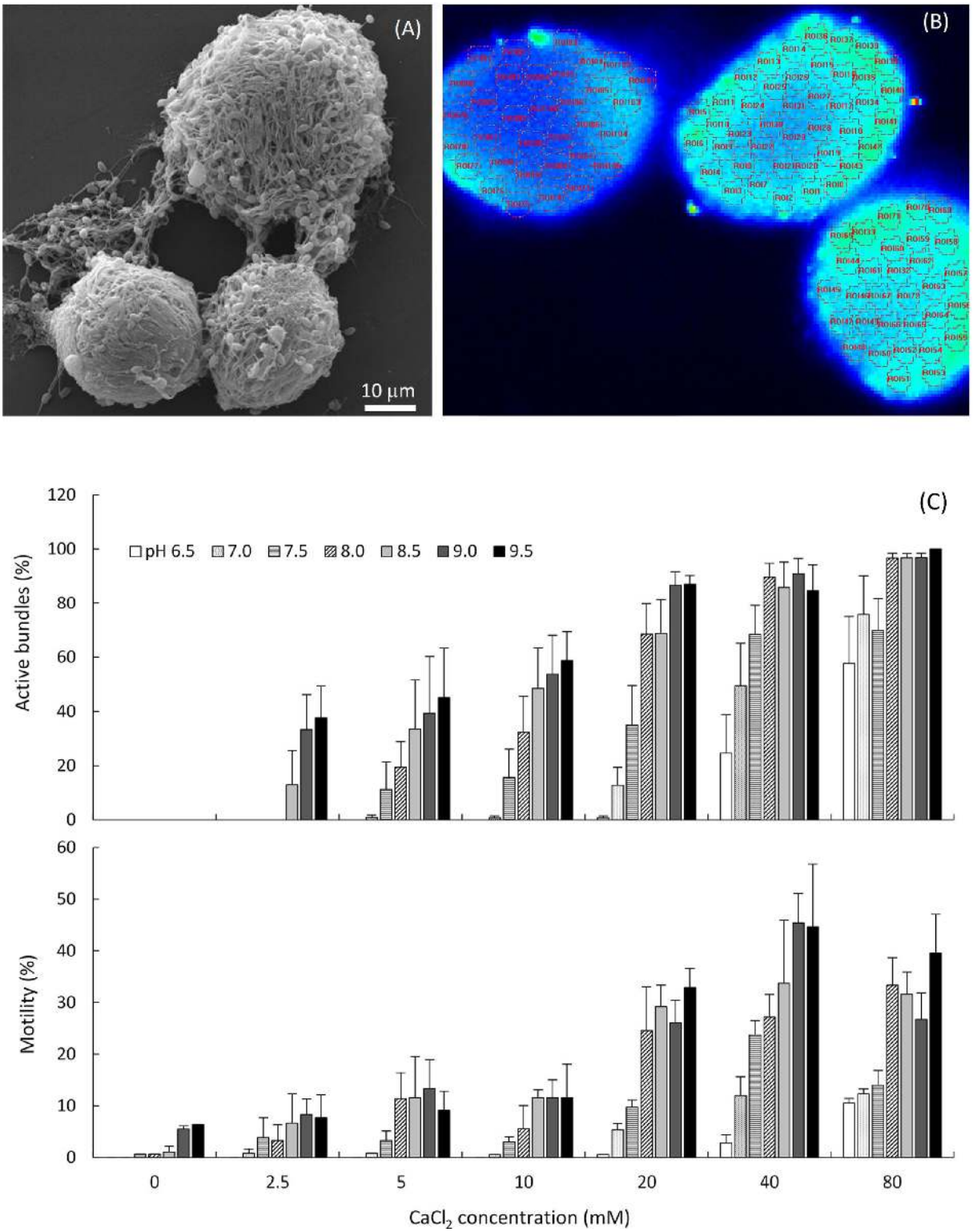

Figure 1. Sperm bundles from Redtail Splitfin. (A) Scanning electron micrographs (SEM) of three sperm bundles. (B) Selection of 105 regions of interest on three bundles loaded with Fura-2 AM and used for Ca^{2+} imaging experiments. Each region of interest can represent multiple sperm cells. (C) Percent activated bundles (top panel) and sperm motility (bottom panel) at 30 s after treatment with different combinations of pH and $CaCl_2$ concentrations. Data are presented as mean ± SEM of five males for the activated bundles and three males for the motility.

Fish husbandry

Protocols for the use of animals were reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee (Baton Rouge, LA). The Redtail Splitfin used in this study were maintained and bred by H. Grier [34], and transported at 6–12 months old from Florida to the Aquatic Germplasm and Genetic Resources Center (AGGRC) at the Louisiana State University Agricultural Center (Baton Rouge). The fish were about 2 years old prior to experiments. About 200 fish were cultured indoors at 22–26°C with a 14 h:10 h (light: dark) photoperiod in four aerated tanks within an 800-l recirculating system and fed twice daily with tropical flakes (Pentair Aquatic Eco-systems, FL) and twice weekly with brine shrimp (Sally's Frozen Brine Shrimp^{TM}, San Francisco Bay Brand, CA). Males were maintained at a 2:1 ratio with females in each tank until 2 days before experiments. Additional water quality parameters were monitored weekly and held within acceptable ranges including dissolved oxygen (>6.0 mg/l), pH (7.0–8.0), ammonia (0–1.0 mg/l), and nitrites (0–0.8 mg/l).

Collection of sperm bundles and free sperm

Fish were anesthetized with 0.03% MS-222, and body length and weight were measured. Sperm cells of Redtail Splitfin are concentrated in the anterior section of the testes. This section was dissected, rinsed, weighed, and placed in 100 μl of extender solution on a glass slide, and gently crushed with angled spade-tip forceps. Sperm bundles were released into the extender and collected with a pipette into a 1.5-ml centrifuge tube as bundle suspension. The extender was NaCl solution at 300 mOsmol/kg (pH = 7.0). No ions except for Na^+ and Cl^- were used in the extender to avoid ionic interaction effects. Because the effects of pH would be tested in experiments by manipulation with Tris-HCl, the pH of extender was not buffered. Our previous studies showed sperm of Redtail Splitfin had highest motility at approximately 300 mOsmol/kg. The standard length of fish used was 35.6 ± 0.8 mm (mean ± SEM), the body wet weight was 1.45 ± 0.11 g, and the testes weight was 18.8 ± 6.0 mg.

To collect free sperm released from dissociated bundles, the anterior sections of the testes (in 100 μl of extender solution) were gently pressed five times using a 1-ml Dounce tissue grinder and its matching pestle. Free sperm and undissociated sperm bundles were released into the extender and collected with a pipette into a 1.5-ml centrifuge tube as free-sperm suspension.

Evaluation of bundle dissociation and free sperm activation

When sperm within bundles were activated, quiescent bundles began to dissociate. Evaluation of bundle dissociation activities reflects the activation levels of sperm within bundles [35]. To activate sperm within bundles, suspensions (1–4 × 10^6 bundles/ml) were mixed with activation solutions at a ratio of 1:4 (bundle suspension: activation solution) on the base of a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) with a coverslip placed on top. The Ca^{2+} activation solutions were 0, 2.5, 5, 10, 20, 40, and 80 mM of $CaCl_2$ mixed with 150, 145, 140, 135, 120, 90, and 30 mM NaCl to yield constant osmolality levels (300 ± 30 mOsmol/kg). The pH of each Ca^{2+} activation solution was adjusted by addition of 5 mM of Tris-HCl at pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5. Activation was observed by use of a dark-field (×200 magnification) microscope (CX41, Olympus Corporation, Tokyo, Japan) coupled with a computer-assisted sperm analysis (CASA) system (HTM-CEROS, version 14 Build 013, Hamilton Thorne Biosciences, MA). A total of 10–15 bundles distributed across a viewing area (with a total of four viewing areas in each observation) were observed at 1 min after mixing with activation solutions and classified as "quiescent bundles" or "active bundles" by evaluating morphology of the bundles and motion of sperm within the bundles. In quiescent bundles, ≤3 sperm attached on the bundles were vibrating in place, and no sperm within the bundles were swimming away. In active bundles, >3 sperm attached on the bundles were vibrating in place, or ≥1 sperm within the bundles was swimming away. The percentage of active bundles was used to evaluate activation levels of sperm within the bundles.

The motility of free sperm (released from bundles) was measured by use of the CASA system. Based on preliminary observations, CASA cell detection was set at 25 pixels for the minimum contrast and 6 pixels for the minimum cell size. In addition, a total of 100 frames were captured at 1–2 min after mixing with activation solutions in each measurement with a frequency of 60 frames/s. Sperm with an average measured path velocity (VAP) of >20 μm/s were counted as motile, according to preliminary trials showing that sperm with VAP < 20 μm/s were usually not detected as motile by the investigator with naked eye. Three to four measurements from different viewing areas were applied and averaged for each observation. After capture, videos were reviewed and tracks with error readings (immotile sperm detected as motile by mistake of the CASA system) were eliminated manually.

Calcium imaging analysis

It was estimated that pH levels were 7.0–7.5 for Redtail Splitfin testicular fluid and 8.0–8.5 for fluid within the female reproductive tract [16]. Suspensions of bundles or free sperm from each male were adjusted to approximately 1 ml with calcium imaging buffers: (NaCl solution at 300 mOsmol/kg) at pH 7.5 (by 5 mM Tris-HCl) for *Experiment 2* to simulate pH levels within the male reproductive tract, pH 7.0 (without Tris-HCl) for *Experiment 3* to evaluate the effects of pH by adjusting pH levels with different Tris-HCl buffers, and pH 8.5 (by 5 mM Tris-HCl) for *Experiment 4* to simulate pH levels within the female reproductive tract. The suspensions were mixed with Fura-2 acetoxymethyl ester (Fura-2 AM) at a final concentration of 2 μM and incubated for 30 min at room temperature in the dark. After incubation, suspensions were centrifuged for 10 s (approximately 800 × *g*) and resuspended with 0.5 ml of calcium imaging buffer. Twenty microliters of this suspension were pipetted onto a 35-mm glass-bottom culture dish (Cellvis, CA), followed by addition of 2.5 ml of buffer. The culture dishes were coated with 200 μl of poly-D-lysine (2.5 × 10^{-6} g/ml) for recording of free sperm to avoid sperm drift during imaging. Samples were observed at ×800 magnification with a fluorescence microscope equipped with a ×40 water immersion objective.

Measurement of intracellular Ca^{2+} signals was performed using a dual-excitation fluorimetric imaging system (TILL-Photonics) controlled by TILLvisION software (Thermo Fisher Scientific, MA). A total of 50–100 regions of interest (each region represented multiple sperm cells) on the surface of sperm bundles (Figure 1B) or 20–50 head areas of free sperm (Supplemental Figure S1) were tagged for imaging analysis. Two to three bundles were used in each experiment. The Fura-2 AM loaded bundles or free sperm were excited at 340/380 nm wavelengths and emission recorded at 540 nm every 2 s and computed into relative ratio units of the fluorescence intensity ($F_{340}/_{380}$). A baseline was recorded prior to bundle (or free sperm) treatments and normalized to 0.5 ratio for comparison between groups. The ratios of baseline fluorescence ($F_{340}/_{380}$)

that were not within 0.4–0.8 (before normalization) were eliminated, because our preliminary studies indicated leakage across the cell membrane at ratios >0.8 and insufficient loading of Fura-2 AM at ratios <0.4. To reduce variation in the ratios, we utilize Fura-2 AM loaded cells within a 90-min window. Data were expressed as averages from ≥50 regions of interest for sperm bundles or ≥20 free sperm cells. Each experiment was performed with bundles (or free sperm) from three different males and was replicated three times.

Experiment 1: Effect of extracellular Ca^{2+} and pH on sperm activation

Bundles were stimulated with $CaCl_2$ concentrations of 0, 2.5, 5, 10, 20, 40, and 80 mM, at pH of 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5 for each $CaCl_2$ concentration. The percentage of active bundles and motility of free sperm was recorded at 30 s after mixing with the activation solutions. Five males as five replicates were used for observations of bundle dissociation, and three males were used for motility of free sperm.

Experiment 2: Effect of extracellular Ca^{2+} on intracellular Ca^{2+} signals

Bundle or free-sperm suspensions (20 μl) were mixed with 2.5 ml of calcium imaging buffer (pH 7.5 by 5 mM Tris-HCl) in culture dishes. Baseline recordings were obtained for ≥30 s prior to addition of 0.3, 0.6, 1.3, 3.2, 6.3, 12.7, and 31.9 μl of 4 M $CaCl_2$ solution to yield final Ca^{2+} concentrations of 0.5, 1, 2, 5, 10, 20, and 50 mM. As a positive control, ionomycin (1 μM) was added to bundle suspensions with 2 mM $CaCl_2$. For each Ca^{2+} concentration, sperm bundles from three different males were used as replicates.

To exclude the effect of autofluorescence on the Ca^{2+} signals generated by Fura-2 AM, $CaCl_2$ with a final concentration of 20 mM was added into bundle suspensions (pH 7.5 by Tris-HCl) without loading of Fura-2 AM, and the imaging was observed for 3 min to evaluate background fluorescence. Areas outside sperm bundles (loaded with Fura-2 AM) were tagged as regions of interest, $CaCl_2$ with a final concentration of 20 mM was added to the suspensions (pH 7.5 by Tris-HCl), and the imaging was observed for longer than 3 min.

Experiment 3: Effect of extracellular pH on intracellular Ca^{2+} signals

Bundle suspensions (20 μl) were mixed with 2.5 ml of calcium imaging buffer (pH 7.0 without Tris-HCl) and 2 mM $CaCl_2$ (within the normal physiological range of calcium concentration in fish semen) in culture dishes. Baseline recordings were obtained for ≥30 s prior to adjusting the pH to 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5 by addition of 5 mM Tris-HCl. For each pH, sperm bundles from three different males were used as replicates.

To determine the Ca^{2+} sources for the intracellular Ca^{2+} signals, 1 μM thapsigargin was used to deplete internal Ca^{2+} stores in the endoplasmic reticulum (ER). After a 30-min incubation, bundle suspensions (20 μl) were mixed with 2.5 ml of calcium imaging buffer (pH 7.0 without Tris-HCl) with or without addition of 1 mM $CaCl_2$ to yield four combinations: (1) Ca^{2+} with thapsigargin, (2) Ca^{2+} without thapsigargin, (3) thapsigargin without Ca^{2+}, and (4) no thapsigargin and no Ca^{2+}. Basal fluorescence of Fura-2 AM was recorded for ≥30 s prior to addition of 5 mM Tris-HCl solutions at pH 8.5. To demonstrate the effects of alkalinity (pH 8.5) on Ca^{2+} influx, a relatively low concentration of extracellular $CaCl_2$ (1 mM) was used.

Experiment 4: Effect of Ca^{2+} channel blockers on intracellular Ca^{2+} signals

Calcium channel blockers $CdCl_2$ (final concentration of 200 μM), $NiCl_2$ (300 μM), ruthenium red (10 μM), nimodipine (30 μM), verapamil (30 μM), mibefradil (40 μM), NNC 55–0396 (10 μM), $GdCl_3$ (100 μM), SKF-96365 (100 μM), methoxyverapamil (D600, 30 μM), ω-Conotoxin MVIIC (2 μM), bepridil (50 μM), and 2-APB (300 μM) were used to identify the Ca^{2+} channels involved in sperm motility activation. Bundles or free sperm were pretreated with each inhibitor for 20 min prior to sperm activation. Bundle or free-sperm suspensions (20 μl) were mixed with 2.5 ml of calcium imaging buffer (pH 8.5 by 5 mM Tris-HCl) in culture dishes. Basal fluorescence of Fura-2 AM was recorded for ≥30 s prior to addition of $CaCl_2$ solutions for a final concentration of 1 mM. To observe the effects of channel blockers, a relatively low concentration of $CaCl_2$ (1 mM) was used to control influx. For each blocker, sperm bundles from three different males were used as replicates.

Data analysis

Statistical analyses were performed using SAS 9.4 (SAS Institute, NC). Differences in the effects of $CaCl_2$ concentration and pH on bundle dissociation and motility were assessed by one-way ANOVA with Tukey's multiple comparisons test, or Kruskal-Wallis nonparametric ANOVA (PROC NPAR1WAY) when assumptions for normality and homoscedasticity were not met. Differences in the effects of $CaCl_2$ concentration and pH on intracellular Ca^{2+} were assessed by one-way ANOVA with Tukey's multiple comparisons test. Data were reciprocal-transformed when assumptions for normality and homoscedasticity were not meet. Peak Ca^{2+} signals from channel blocker experiments were compared with the control group using a *t*-test. Differences were considered significant when $P < 0.05$ (*P*-values are reported as "<0.0001" instead of actual values when below 0.0001).

Results

Experiment 1: Effect of extracellular Ca^{2+} and pH on sperm activation

At each pH level (6.5–9.5), the percentage of active bundles increased with $CaCl_2$ concentration ($0.0002 < P < 0.0206$) (Figure 1C). For example, at all pH levels no bundle dissociation was observed with 0 mM $CaCl_2$. At pH 6.5, ≤1% of bundles were active in 2.5–20 mM $CaCl_2$, whereas 25% were active in 40 mM $CaCl_2$ and 58% were active in 80 mM $CaCl_2$. At pH 7.0, the active bundles were ≤1% in 2.5–10 mM $CaCl_2$, but increased to 13–76% in 20–80 mM $CaCl_2$. At pH 7.5 and 8.0, bundle dissociation was only observed in 5–80 mM $CaCl_2$. At pH 8.5–9.5, the active bundles were ≤38% in 2.5 mM $CaCl_2$, but ≥97% in 80 mM $CaCl_2$. At each $CaCl_2$ concentration (0 mM was not statistically tested because no bundle dissociation was observed), the percentage of active bundles increased with pH ($0.0001 < P < 0.0012$) (Figure 1C). For example, with 2.5 mM $CaCl_2$, bundle dissociation was only observed at pH 8.5 (13%), 9.0 (33%), and 9.5 (38%). In 5 to 20 mM $CaCl_2$, the percentage of active bundles increased from 0–13% at pH 6.5–7.0 to 39–87% at pH 9.0–9.5. In 40–80 mM $CaCl_2$, the percentage of active bundles was 25–58% at pH 6.5–7.0, but ≥85% at pH ≥ 8.0.

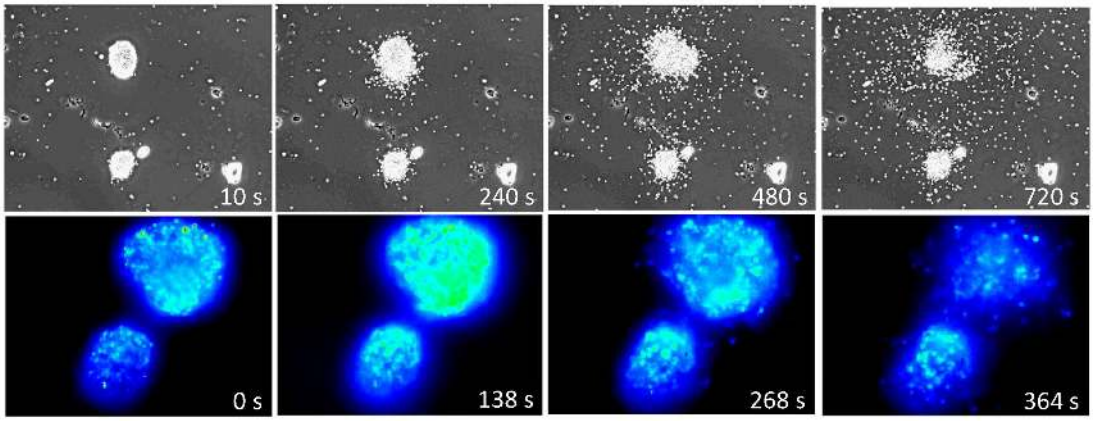

Figure 2. Dissociation of sperm bundles from Redtail Splitfin. The top panel: dark-field images (×200 magnification) of two bundles at 10, 240, 480, and 720 s (from left to right) after treatment with 20 mM $CaCl_2$ condition. The bottom panel: fluorescence images of two bundles at pH 8.5 captured at 0, 138, 268, and 364 s after addition of 1 mM $CaCl_2$. Note the increase in intracellular Ca^{2+} signal (marked by the increase of light-blue coloration) in the second panel (138 s) followed by bundle dissociation and sperm motility.

At each pH level (6.5–9.5), the motility increased ($0.0001 < P < 0.0124$) with $CaCl_2$ concentration (Figure 1C). For example, with increasing $CaCl_2$ concentrations from 2.5 to 80 mM, the motility increased from 0–8% across all pH levels to 11–40%. At each $CaCl_2$ concentration (except for 2.5 mM), the motility increased ($0.0008 < P < 0.0378$) with pH level (Figure 1C). In the absence of extracellular $CaCl_2$, the motility increased from <1% at pH 6.5–8.5 to about 6% at pH 9.0–9.5. In 2.5 to 10 mM $CaCl_2$, the motility increased from <1% at pH 6.5–7.0 to about 10% at pH 8.0–9.5. In 40 mM $CaCl_2$, the motility increased from <12% at pH 6.5–7.0 to 45% at pH 9.0–9.5. Dark-field microscope imaging showed the effect of extracellular $CaCl_2$ on bundle dispersion (Figure 2, Supplementary Movie S4). Bundles were quiescent and undissociated, and no motility of free sperm was observed at 10 s after addition of 20 mM $CaCl_2$ to the extracellular buffer at pH 7.5. At 240 s, sperm within bundles were activated but most of them were still attached to the bundles. At 480 s, sperm swam away from bundles, resulting in motile free sperm. At 720 s, additional sperm moved away from the bundles and the motility of free sperm increased.

Experiment 2: Effect of extracellular Ca^{2+} on Ca^{2+} influx

Addition of 0.5–50 mM extracellular $CaCl_2$ increased intracellular Ca^{2+} in a concentration-dependent manner with peak at approximately120 s (Figure 3A). The Ca^{2+} influx significantly increased ($P < 0.0001$) with an increase in extracellular Ca^{2+} (Figure 3B). Intracellular Ca^{2+} levels were 633–1940% higher with 10–50 mM extracellular Ca^{2+} compared to 0.5–2 mM. The increase caused by 50 mM $CaCl_2$ was 74% of that stimulated by 1 μM ionomycin with 2 mM extracellular $CaCl_2$ ($P > 0.05$). To confirm that the intracellular Ca^{2+} signals were from sperm cells rather than from some other bundle components, free sperm were stimulated with increasing $CaCl_2$ concentrations. The addition of extracellular $CaCl_2$ increased Ca^{2+} influx of free sperm in a concentration-dependent pattern (Figure 3C and D, Supplemental Figure S1) similarly to those of bundles, with the intracellular Ca^{2+} levels higher with 10–50 mM $CaCl_2$ compared to those with 0.5–2 mM $CaCl_2$ ($P < 0.0001$).

Next, we excluded the effect of autofluorescence as a cause of the Ca^{2+} signals generated by Fura-2 AM. In the absence of Fura-2 AM loading, there were no changes in the fluorescence signals of bundles before or after addition of 20 mM $CaCl_2$ (pH 7.5) (Figure 4A), but bundles loaded with Fura-2 AM showed the increase in Ca^{2+} signals. When examining background fluorescence, the Ca^{2+} signals from regions of outside bundles also remained unchanged before and after addition of $CaCl_2$ (Figure 4B).

Experiment 3: Effect of extracellular pH on Ca^{2+} influx

Intracellular Ca^{2+} increased with extracellular pH ($P < 0.0001$). With 2 mM extracellular $CaCl_2$, pH levels of 6.5–7.5 failed to significantly increase intracellular Ca^{2+} signals (Figure 3E and F). However, a pH level of pH 8.0 caused a significant increase in intracellular Ca^{2+} levels of sperm within bundles (Figure 3E and F). Intracellular Ca^{2+} increases at pH 8.5–9.5 were higher than at pH 6.5–8.0 ($P < 0.0001$) (Figure 3E and F). Dissociation of sperm bundles was observed after changes of fluorescent signals for intracellular Ca^{2+} (Figure 2, Supplementary Movie S5). To confirm that the Ca^{2+} signals were due to influx and not release of Ca^{2+} from the ER, we performed experiments using extracellular Ca^{2+}-free conditions and depletion of intracellular Ca^{2+} stores in the ER by pretreatment of bundles with 1 μM thapsigargin for 30 min. Intracellular Ca^{2+} increased in response to stimulation of pH 8.5 (with 1 mM extracellular $CaCl_2$), with or without pretreatment with thapsigargin (Figure 5A). In the absence of extracellular $CaCl_2$, stimulation with pH 8.5 failed to increase intracellular Ca^{2+} with or without pretreatment with thapsigargin (Figure 5A). Addition of 1 mM $CaCl_2$ to the extracellular buffer increased Ca^{2+} influx after stimulation with pH 8.5 under Ca^{2+}-free conditions (Figure 5B). These results indicated that increases in intracellular Ca^{2+} triggered by alkalinization were due to influx from extracellular sources.

Experiment 4: Effect of different Ca^{2+} channel blockers on Ca^{2+} influx

Because Ca^{2+} influx triggers sperm activation and motility, we tested the effect of Ca^{2+} channel blockers as a means to identify the pathway by which Ca^{2+} enters the cell. In this experiment, only $CdCl_2$ caused a significant reduction (approximately25%) ($P = 0.0304$) in the Ca^{2+} influx (Figure 5C). Pretreatment of bundles with $NiCl_2$ ($P = 0.6896$), ruthenium red ($P = 0.5993$), $GdCl_3$ ($P = 0.4529$), SKF-96365 ($P = 0.5861$), nimodipine ($P = 0.6851$), verapamil

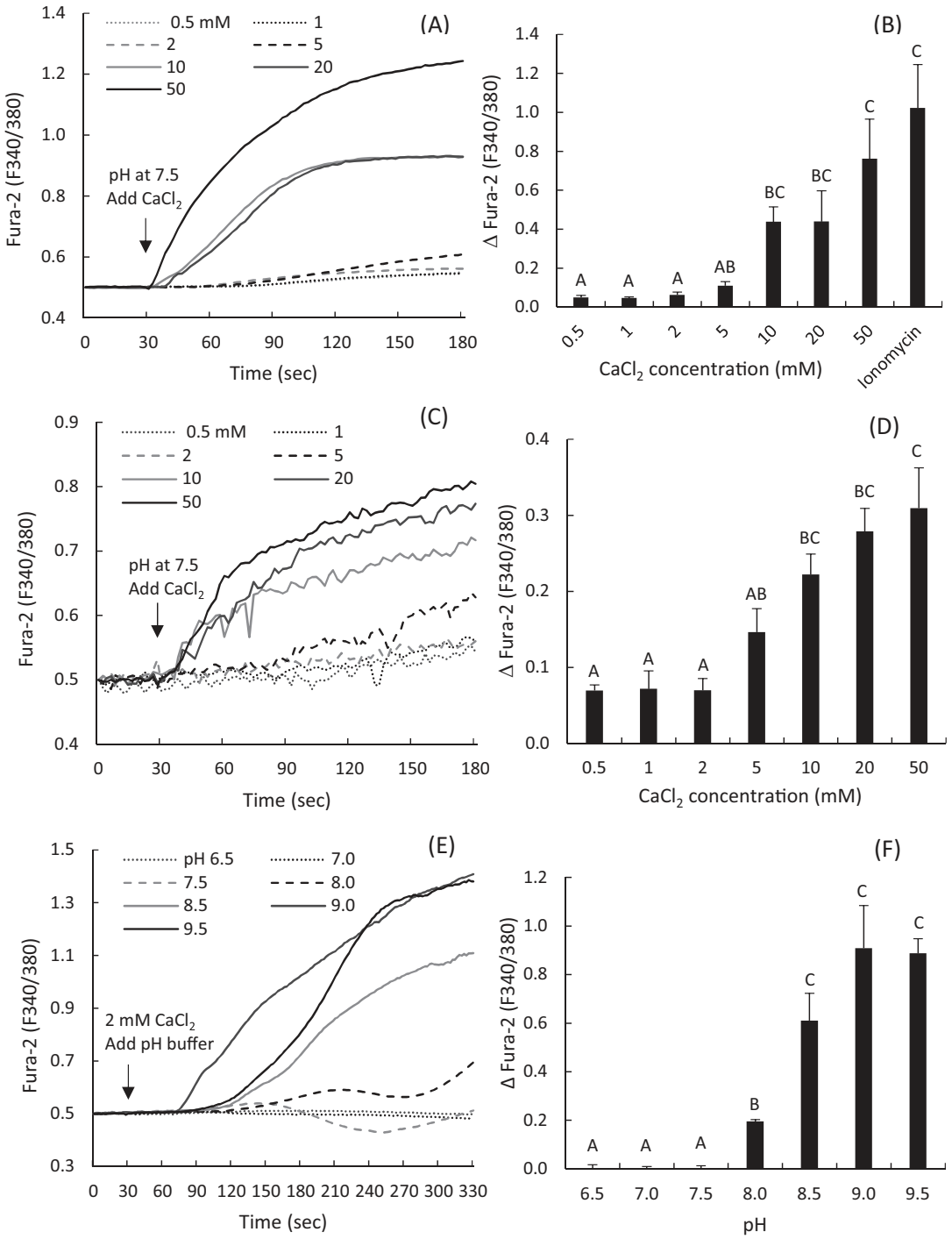

Figure 3. Increases in intracellular Ca^{2+} concentration of sperm from Redtail Splitfin. (A) Average Ca^{2+} signals of sperm within bundles during stimulation with different $CaCl_2$ concentrations at pH 7.5. (B) Average peak increases of sperm within bundles for each $CaCl_2$ concentration compared with 1 μM ionomycin. (C) Average Ca^{2+} signals of dissociated single sperm cell from bundles during stimulation with different $CaCl_2$ concentrations at pH 7.5. (D) Average peak increases of dissociated single sperm cells from bundles for each $CaCl_2$ concentration. (E) Average Ca^{2+} signals of sperm within bundles during stimulation (0–330 s) with different pH in the presence of 2 mM $CaCl_2$. (F) Average peak increases of sperm within bundles for each pH level. Bars represent means ± SEM; n = 50–150 regions of interest per male from three males. Values sharing letters were not significantly different.

(P = 0.6087), thapsigargin (P = 0.8053), D600 (P = 0.3742), mibefradil (P = 0.9767), NNC 55–0396 (P = 0.1941), ω-Conotoxin MVIIC (P = 0.8560), bepridil (P = 0.1299), and 2-APB (P = 0.2333) all failed to inhibit Ca^{2+} influx compared to control bundles. Intracellular Ca^{2+} began to rise at about 100 s in $GdCl_3$, mibefradil, NNC 55–0396, and bepridil, which was delayed in comparison to the other blockers and the control group, which began to increase at 50 s (Supplemental Figure S2). These blockers also failed to inhibit influx into free sperm cells (Supplemental Figure S3).

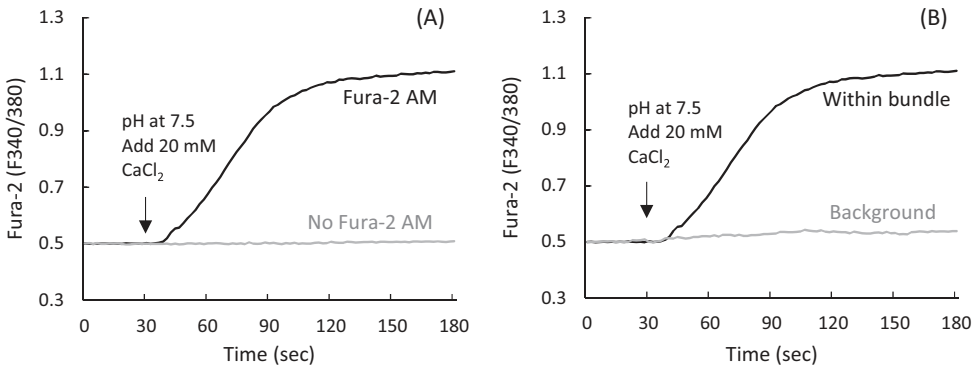

Figure 4. Effect of autofluorescence and background on Ca^{2+} signals. (A) Increase in intracellular Ca^{2+} by 20 mM $CaCl_2$ (pH = 7.5) in bundles with Fura-2 AM loading (black line) and without (gray line, autofluorescence). (B) Increase in intracellular Ca^{2+} by 20 mM of $CaCl_2$ (pH = 7.5) in bundles (black line) and from background areas outside of bundles (gray line).

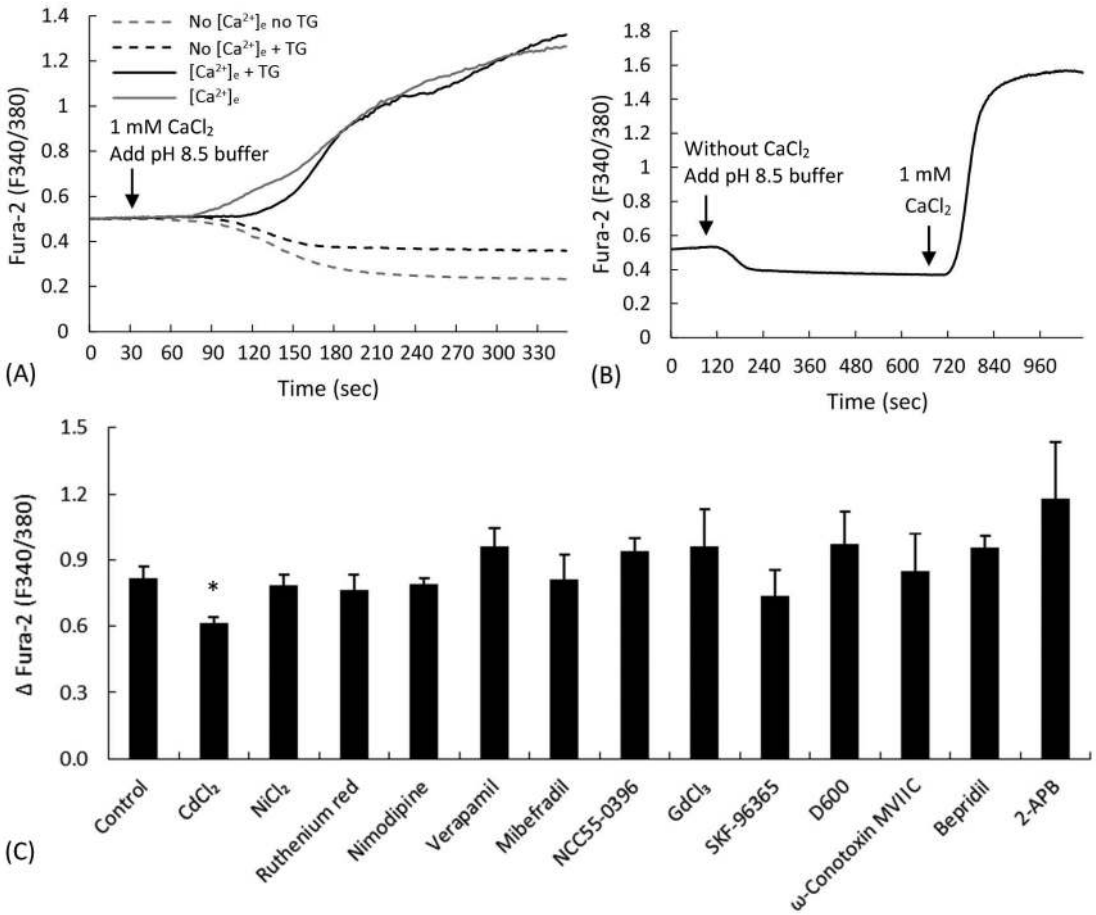

Figure 5. Intracellular Ca^{2+} increase is dependent on extracellular sources and the increases were not inhibited by major Ca^{2+} channel blockers. (A) Thapsigargin (TG, 1 μM) was added to bundle suspensions with or without 1 mM $CaCl_2$ to deplete intracellular Ca^{2+} stores prior increase of pH to 8.5. (B) Addition of 1 mM $CaCl_2$ to the extracellular buffer increased Ca^{2+} influx after stimulation with pH 8.5 under Ca^{2+}-free conditions. (C) Bundles were pretreated the respective blockers ($CdCl_2$ (200 μM), $NiCl_2$ (300 μM), ruthenium red (10 μM), nimodipine (30 μM), verapamil (30 μM), mibefradil (40 μM), NNC 55–0396 (10 μM), $GdCl_3$ (100 μM), SKF-96365 (100 μM), methoxyverapamil (D600 30 μM), ω-Conotoxin MVIIC (2 μM), bepridil (50 μM), or 2-APB (300 μM) for 20 min prior to stimulation by extracellular exposure to 1 mM $CaCl_2$ stimulation and pH 8.5. Bars represent means ± SEM; n = 50–150 regions of interest per male from three males. Only $CdCl_2$ treatment (asterisk) produced a significant reduction in relation to the control group (without blocker) and blockers.

Discussion

Sperm bundles are believed to be adaptations to efficiently transfer male gametes into the female [36, 37]. However, it is unclear how sperm bundles dissociate or sperm within bundles are activated after this transfer to the female during natural spawning. Mechanisms of sperm motility activation have been elucidated in some oviparous fishes, but remain unknown in viviparous species, and the presence of sperm bundles complicates such studies. Intracellular Ca^{2+} has been proven important in regulating sperm motility of oviparous fishes; however, its role in viviparous species is unknown. Using the viviparous fish Redtail Splitfin as a model, we established methodologies to investigate intracellular Ca^{2+} signaling of sperm within bundles. We found that sperm bundle dissociation, sperm motility, and Ca^{2+} influx into cells increased with elevations in extracellular Ca^{2+} at alkaline pH, and that the Ca^{2+} influx could be partially blocked by $CdCl_2$, but not by other channel blockers known to have an effect in fish and mammalian sperm. Overall, this is the first report of fish sperm activation by Ca^{2+} influx mediated by extracellular alkalinization.

This is different from osmolality-dependent activation of oviparous fish sperm, likely because sperm from viviparous species are activated in an isotonic environment within the female reproductive tract, and thus osmotic changes are not critical for motility activation. As such, what is the key to activation of sperm cells of viviparous fishes? In the present study, there was a direct correlation between sperm motility and percent bundle dissociation with increases in extracellular Ca^{2+} and pH in Redtail Splitfin. It is possible that upon spawning, there is an increase in Ca^{2+}, pH, or both in the female reproductive tract that activates sperm within bundles that result in bundle dissociation. This hypothesis is consistent with pH levels of 7.0–7.5 of Redtail Splitfin testicular fluid and pH of 8.0–8.5 in the female reproductive tract fluid [16]. Unfortunately, because of the limited volume of the fluid from the testes and female reproductive tract (<1 μl) [38], the Ca^{2+} concentration could not be determined. However, the concentration of Ca^{2+} (2.5–80 mM) in our study is within normal physiological range of seminal fluid of large-sized fishes (0.7–82 mM in cyprinids) [39]. In the absence of $CaCl_2$, no bundles were dissociated across all pH levels, but 1–6% motility was observed at pH 7.5–9.5, indicating extracellular Ca^{2+} is critical but not prerequisite to sperm activation of Redtail Splitfin. Motility in the absence of $CaCl_2$ could be caused by release of intracellular Ca^{2+} from damaged plasma membranes or residual Ca^{2+} from semen. In the present study, bundle dissociation and sperm motility were observed at 1–2 min after mixing of samples with activation solutions. Future studies could include observation of bundle dissociation and sperm motility for longer duration (>1 h) because it could take hours for bundles to dissociate in the female reproductive tract.

During sperm activation of oviparous fish, Ca^{2+} can act extracellularly as a divalent cation to mask membrane potential [12], or enter the cell to function as a second messenger [11]. To investigate whether Ca^{2+} influx and signaling was involved in bundle dispersion and sperm activation, we established methodologies to study intracellular Ca^{2+} signaling of sperm cells within bundles. A variety of methods have been used to study intracellular Ca^{2+} of free sperm, such as flow cytometry [40], cell imaging [11, 41], and fluorescence spectrophotometry [42]. However, the presence of sperm bundles represents an obstacle for these methods. For example, flow cytometers require individual passage of cells through a laser beam [33], which is not feasible for intact bundles. Redtail Splitfin [35] and Guppy sperm bundles (our unpublished observation) are 30–150 μm in diameter, but most flow cytometers have a maximum size for particle passage of 40–80 μm. Although bundles from poeciliids can be dissociated by physical pressure and suitable activation solutions prior to loading into a flow cytometer [43], most of bundles from goodeids cannot be dissociated [16].To detect Fura-2 AM fluorescence from sperm within bundles, >20 regions of interest (each region represents multiple cells) were selected for each intact bundle with a total of two to three bundles. Using this method, we detected for the first time intracellular Ca^{2+} signals arising from viviparous fish sperm bundles. These signals were confirmed in dispersed free sperm cells. The possibility of autofluorescence of bundles or sperm cells was also excluded. Based on these observations, the methodologies established are viable for studying of intracellular Ca^{2+} signals of sperm within bundles, and can be applied to sperm bundles of other species.

The increases in intracellular Ca^{2+} and percentage of bundle dissociation caused by addition of extracellular Ca^{2+} were concentration dependent. This was also observed in dispersed free sperm. Thus, it appears that increases in intracellular Ca^{2+} activate sperm from Redtail Splitfin. Intracellular Ca^{2+} signals are a critical second messenger that regulates sperm motility in invertebrates such as sea urchins [44], and vertebrates such as mammals [45] and fishes. In oviparous fishes, the prerequisite for intracellular Ca^{2+} signaling during sperm activation is seen in freshwater species such as Common Carp [11], Sterlet Sturgeon (*Acipenser ruthenus*) [46], salmonids [47, 48], saltwater species such as Atlantic Croaker (*Micropogonias undulatus*) [49], Pacific herring (*Clupea pallasii*) [42], Stone Flounder, Grass Puffers, and Panther Puffer [13], and euryhaline species such as Mozambique Tilapia (*Oreochromis mossambicus*) [50]. In viviparous fishes, intracellular signaling during sperm activation is known only in Guppy, in which sperm bundles are dissociated by extracellular Ca^{2+} without changes in the intracellular levels, suggesting an extracellular mechanism without influx in this species [15]. This is different from the intracellular Ca^{2+} level changes observed in Redtail Splitfin, implying separate evolutionary paths involving regulation of isotonic sperm activation from the poeciliids and goodeids. This is consistent with the independent evolution of viviparity of the family Poeciliidae and Goodeidae within Cyprinodontiformes [17], but also raises questions on how internal fertilization evolved in these species and the diversity present in regulatory mechanisms of sperm activation among viviparous fishes.

The activation of mammalian sperm is triggered by Ca^{2+} influx under alkaline conditions through CatSper channels [41, 51, 52]. In fish, the effects of pH on sperm activation have not been found to be as pronounced as the effects of osmotic pressure and ions [20], and no Ca^{2+} influx mediated by pH changes has been previously reported. Interestingly, we found that the percentage of bundle dissociation and sperm motility each increased with alkaline conditions. At pH > 8.0, there was a significant increase in Ca^{2+} influx even at low extracellular Ca^{2+} levels of ≤2 mM (Figure 3F), but with no influx at pH 7.0 (neutral) or 6.5 (acidic) conditions. These observations indicate that sperm activation of the Redtail Splitfin is regulated by extracellular pH via Ca^{2+} influx and signaling. Further studies are needed to elucidate levels of intracellular alkalinization.

To investigate the source of the Ca^{2+} signals, sperm bundles were incubated with thapsigargin to deplete intracellular Ca^{2+} stores in the ER prior to stimulation by pH 8.5. Pretreatment of sperm with thapsigargin did not inhibit the Ca^{2+} signals compared to control. Moreover, without extracellular Ca^{2+} no increases of intracellular

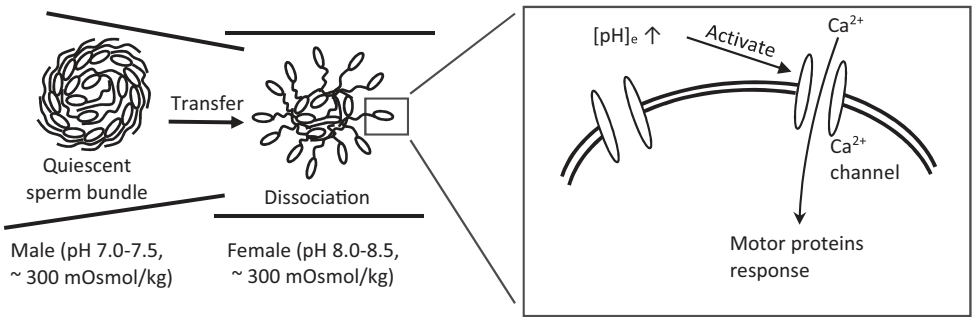

Figure 6. Schematic hypotheses of major signaling events involved in dissociation of sperm bundles from Redtail Splitfin during natural spawning. Upon transfer of sperm bundles from the male to the female reproductive tract, the pH of fluid around sperm cells increases from 7.0–7.5 to 8.0–8.5 without osmotic shock. The pH increase activates Ca^{2+} channels, inducing extracellular Ca^{2+} influx as a second messenger to activate motor proteins for sperm motility. Activation of sperm within bundles results in dissociation of bundles. The involvement of intracellular pH needs to be further studied.

Ca^{2+} stimulated by alkalinization were observed. These findings indicate that extracellular alkalinization induced the Ca^{2+} influx from extracellular sources rather than from release of intracellular stores. Interestingly, a decrease in intracellular Ca^{2+} level was observed at pH 8.5 under extracellular Ca^{2+}-free conditions (Figure 5B). It is possible that this reduction was due to the ion being removed from the cell (e.g. via Ca^{2+} pumps) or sequestration into intracellular Ca^{2+} stores. Upon addition of 1 mM $CaCl_2$ to the Ca^{2+}-free experiment, the Ca^{2+} influx could be observed once again, which confirms that the intracellular signals were due to influx. We hypothesized that extracellular Ca^{2+} was transported into cells by Ca^{2+} channels down their concentration gradient.

As such, we tested all known Ca^{2+} channel blockers and surprisingly, none of them effectively inhibited Ca^{2+} influx into sperm within bundles except for $CdCl_2$ (which displayed 25% inhibition). Furthermore, these blockers also failed to inhibit influx into free sperm cells (Supplemental Figure S3), indicating that the bundle structure did not affect the drug action. In Common Carp sperm, Ca^{2+} influx is inhibited by verapamil and D600 [11], which blocks L-type voltage-gated calcium channel types such as Ca_v 1.1, Ca_v 1.2, and Ca_v 1.3 [53, 54], and by ω-Conotoxin MVIIC, a P-, Q-, and N-type blocker that inhibit channel types Ca_v 2.1 and Ca_v 2.2 [55]. Sperm motility in salmonids is inhibited by nimodipine [56]. In Pacific Herring, a Na^+/Ca^{2+} channel promotes Ca^{2+} influx during sperm activation, and is inhibited by bepridil [42]. Osmosis-activated sperm motility of saltwater and freshwater fishes is inhibited by Gd^{3+}, a mechano-sensitive channel blocker [57].

When studied beyond fishes, alkaline pH-induced Ca^{2+} influx is similar to the pH-gated Ca^{2+} influx through CatSper channels in mammalian sperm. A CatSper-like protein was found in some fishes, including Flounders, Medaka (*Oryzias latipes*), Pacific Herring, and Rainbow Trout [58]. CatSper can be blocked by mibefradil and NNC55–0396 [18, 41]; however, all of these drugs failed to inhibit Ca^{2+} influx in sperm of Redtail Splitfin in the present study. Mibefradil is also known to block T-type voltage-gated calcium channels Ca_v 3.1, Ca_v 3.2, and Ca_v 3.3 [59]. Transient receptor potential channels can activate human sperm [60], and are inhibited by ruthenium red [61, 62], Gd^{3+} [63], 2-APB [64], and SKF-96365[60]. Nickel (Ni^{2+}) blocks R-type (Ca_v 2.3) [65] and T-type [66] voltage-gated calcium channels in mammalian cells. Blockers described above have been previously used to identify the pathway for Ca^{2+} influx through all known Ca^{2+} channels in fish sperm and mammalian cells; however, all of these blockers with previously effective concentrations failed to inhibit Ca^{2+} influx into sperm within bundles or into free sperm of Redtail Splitfin. It is unclear why Cd^{2+} was able to partially inhibit Ca^{2+} influx, but it is possible that Cd^{2+} could cause a physical block of the Ca^{2+} channel in question. Cd^{2+} is known to block channels for CatSper, TPC1, TPR6, TPV5, and TPV6 [67]. However, other blockers for these channels showed no effect in the present study. Thus, it appears that a novel Ca^{2+} channel promotes influx to activate sperm of Redtail Splitfin. Because these drugs and their effective doses were developed for mammalian cells, there is the possibility that a different protein structure and molecular origin make these blockers (with previously effective concentrations) ineffective for sperm of fishes such as Redtail Splitfin. The $GdCl_3$, mibefradil, NNC 55–0396, and bepridil might delay channel activation (Supplemental Figure S2); however, this effect needs to be confirmed. Further studies are needed to identify the sequences, structure, and localization of Ca^{2+} channels in Redtail Splitfin sperm via techniques such as RNA sequencing (because no genes related to ion channels have been reported for the Goodeidae family).

Based on our findings, we propose a new regulatory mechanism among those reported for fish sperm activation (Figure 6). Upon spawning of Redtail Splitfin, pH levels around sperm increase due to bundle transfer from the male to female reproductive tract without osmotic shock. The alkaline environment activates Ca^{2+} channels in the plasma membrane to promote Ca^{2+} influx. The increase in intracellular Ca^{2+} functions as a second messenger to activate motor proteins controlling flagella movement. This mechanism found in a viviparous fish from the family Goodeidae is different from the Ca^{2+} activation mechanism proposed for Poeciliidae, although both families belong to the order Cyprinodontiformes. The difference in regulatory mechanism for these two taxa may reflect the independent evolution of viviparity among fishes. This study provides an insight on the uniqueness of sperm motility regulatory systems of viviparous fishes. To date, such regulatory mechanisms for most viviparous fishes are unknown. The methodology established in this study can be used to investigate other viviparous species and other species that utilize sperm bundles in livebearers. Finally, understanding the mechanism of sperm activation can offer a foundation for standardization or optimization of research or commercial reproduction applications using gametes, such as development of germplasm repositories for biomedical and imperiled species, studies of evolution and behavior, or artificial reproduction of ornamental species. Once these Ca^{2+} channels have been identified, in vivo experiments in knockout animals can be performed to validate our findings and determine their physiological impact on fertility.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Figure S1. Selection of regions of interest on 27 dissociated free sperm from bundles of Redtail Splitfin.

Supplementary Figure S2. Effect of Ca^{2+} channel blockers on the $CaCl_2$-induced increase in intracellular Ca^{2+} concentration of sperm bundles. The extracellular solution was buffered with Tris-HCl at pH 8.5 and prepared with Ca^{2+} channel blockers $CdCl_2$ (200 μM), $NiCl_2$ (300 μM), ruthenium red (10 μM), nimodipine (30 μM), verapamil (30 μM), thapsigargin (1 μM), mibefradil (40 μM), NNC 55–0396 (10 μM), $GdCl_3$ (100 μM), SKF-96365 (100 μM), methoxyverapamil (D600 30 μM), ω-Conotoxin MVIIC (2 μM), bepridil (50 μM), and 2-APB (300 μM). Cells were stimulated by addition of 1 mM $CaCl_2$. n = 50–150 regions of interest.

Supplementary Figure S3. Effect of Ca^{2+} channel blockers on intracellular Ca^{2+} signals from dissociated free sperm cells. The extracellular solution was buffered with Tris-HCl at pH 8.5 and prepared with Ca^{2+} channel blockers $CdCl_2$ (200 μM), $NiCl_2$ (300 μM), ruthenium red (10 μM), nimodipine (30 μM), verapamil (30 μM), mibefradil (40 μM), NNC 55–0396 (10 μM), $GdCl_3$ (100 μM), SKF-96365 (100 μM), methoxyverapamil (D600 30 μM), ω-Conotoxin MVIIC (2 μM), bepridil (50 μM), and 2-APB (300 μM). Cells were stimulated by addition of 1 mM $CaCl_2$. n = 20–50 sperm cells.

Supplementary Movie S4. Video captured with a CCD camera on a microscope at dark field (×200 magnification) of two bundles from 10 to 720 s after treatment with 20 mM $CaCl_2$ at pH 7.5. After mixing $CaCl_2$, sperm within bundles are activated. Quiescent bundles start to dissociate and sperm within bundles swam away from bundles. Sperm might stop swimming or slowdown in this video due to resistance of bottom or cover slip of the counting chamber.

Supplementary Movie S5. Fluorescence images of two bundles under pH 8.5 before and after addition of 1 mM $CaCl_2$ at 40 s. Note the increase in intracellular Ca^{2+} signal in the second panel followed by bundle dissociation and sperm motility.

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