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Activation of free sperm and dissociation of sperm bundles (spermatozeugmata) of an endangered viviparous Fish, *Xenotoca eiseni*

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Abstract

Knowledge of sperm motility activation for viviparous fishes has been limited to study of several species in Poeciliidae, and the dissociation of sperm bundles is poorly understood. The goal of this study was to use the endangered Redtail Splitfin (*Xenotoca eiseni*) as a model to investigate the activation of sperm from viviparous fish by study of free sperm and spermatozeugmata (unencapsulated sperm bundles). The specific objectives were to evaluate the effects of: (1) osmotic pressure and refrigerated storage (4 ºC) on activation of free sperm, (2) osmotic pressure, ions, and pH on dissociation of spermatozeugmata, and (3) $CaCl_2$ concentration and pH on sperm membrane integrity. Free sperm were activated in Ca^{2+}-free Hanks' balanced salt solution at 81–516 mOsmol/kg. The highest motility (19 ± 6%) was at 305 mOsmol/kg and swim remained for 84 h. Glucose (300–700 mOsmol/kg), NaCl (50–600 mOsmol/kg), and KCl, $MgCl_2$, and $MnCl_2$ at 5–160 mM activated sperm within spermatozeugmata, but did not dissociate spermatozeugmata. $CaCl_2$ at 5–160 mM dissociated spermatozeugmata within 10 min. Solutions of NaCl-NaOH at pH 11.6 to 12.4 dissociated spermatozeugmata within 1 min. The percentage of viable cells had no significant differences ($P = 0.2033$) among different concentrations of $CaCl_2$, but it was lower ($P < 0.0001$) at pH 12.5 than at pH between 7.0 to 12.0. Overall, this study provided a foundation for quality evaluation of sperm and spermatozeugmata from livebearing fishes, and for development of germplasm repositories for imperiled goodeids.

Keywords

Sperm activation; Sperm bundles; Viviparous fish; Goodeids; *Xenotoca eiseni*; Endangered species; Fish conservation; Sperm cryopreservation; Ca^{2+}; pH

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1. Introduction

In most fish species, sperm are immotile inside the testes and male genital tract, and during the process of natural spawning they become motile upon discharge into the aqueous environment in externally fertilized species, or into the female reproductive tract in internally fertilized species (Cosson, 2010). The activation of sperm is essential for reproduction because within the short motility duration activated sperm are able to reach, bind, and penetrate eggs, and initiate fertilization (Dzyuba and Cosson, 2014). Motility activation is also important for evaluation of sperm quality for induced spawning, development of protocols for sperm cryopreservation, and research activities to address sperm competition. Several physicochemical factors play important roles in inducing motility activation of fish sperm. For most externally fertilized species, sperm motility can be induced by hypotonic solutions in freshwater fishes, and hypertonic solutions in saltwater fishes (Alavi and Cosson, 2006). For euryhaline species, such as Medaka (*Oryzias latipes*) (Yang and Tiersch, 2009a) and Tilapia (*Oreochromis mossambicus*) (Linhart et al., 1999), sperm motility can be activated by hypotonic, isotonic, or hypertonic media. In some species, motility can be activated by electrolytic and non-electrolytic solutions within certain ranges of osmotic pressures (Gallego Albiach, 2013; Morisawa, 2008). In some species, in addition to or as an alternative to osmotic pressure, concentrations of ions are critical to initiate motility. For example, in salmonids, relatively high levels of K^+ concentration are a major inhibitor of sperm motility prior to spawning, whereas Ca^{2+} is antagonistic to this inhibitory effect (Alavi and Cosson, 2006). In addition, pH of the activation media can also influence the triggering of sperm motility, but its effect is not as pronounced as that observed for osmotic pressure and ions (Alavi and Cosson, 2005; Boitano and Omoto, 1991).

The various requirements of environmental osmotic pressure and ions to induce motility activation reflect different environmental adaptations of fishes. Viviparity has evolved repeatedly among fishes, and has been documented in chondrichthyans (40 families), coelacanths (1 montypic family), and several teleosts (13 families) (Wourms, 1981). Viviparous fish employ reproductive adaptations distinct from oviparous species, such as the bearing of live young, maternal-embryo nutrient transfer, and internal fertilization. Internal fertilization is the prerequisite of reproductive success for viviparous species, but sperm from internally fertilized fishes must overcome successive challenges to produce fertilization. The journey begins upon departure from the male and transfer into the female reproductive tract. In internally fertilized fishes, male gametes are delivered to the female by transfer with copulatory organs, which are usually modified pelvic or anal fins, such as claspers in Chondrichthyes (Wourms, 1977), gonopodia in poeciliids, or andropodia (split fin) in goodeids (Meyer and Lydeard, 1993). Male copulatory organs may not deliver the gametes sufficiently into the female reproductive tract to ensure residence. For example, in goodeids, the andropodium does not function as an intromittent organ to insert male gametes into females, but acts as a copulation device to form a pocket around the female urogenital opening, assisting transfer of the gametes (Greven and Brenner, 2010). Thus, the aqueous environment can disturb the transfer process by flushing free gametes away from the female. Spermatozeugmata and spermatophores are believed to be adaptations to facilitate efficient transfer of male gametes into the female (Greven, 2005; Grier et al., 1978), possibly by

resisting flushing effects. However, it is poorly understood how sperm bundles are dissociated and the sperm activated in the female reproductive tract.

Internal fertilization requires acquisition of specific characteristics of sperm such as isotonic activation and relatively long motility duration. For example, in saltwater fishes, sperm of the viviparous shark Banded Houndshark (*Triakis scyllia*) gain motility potential while in the male reproductive tract and remain motile in isotonic (to the uterus fluid) electrolytic solutions for more than 12 h (Inaba et al., 1998). In freshwater species, Poeciliidae, the largest livebearing teleost family (around 136 viviparous species) (Wourms, 1981), is the most studied for patterns of sperm activation among viviparous fishes due to their ornamental value and importance as biomedical research models. In this family sperm motility of Mosquitofish (*Gambusia affinis*) was induced by an isotonic electrolytic solution and suppressed by a non-electrolyte (Morisawa and Suzuki, 1980). Sperm from Green Swordtail (*Xiphophorus helleri*) (Huang et al., 2004b, c; Yang et al., 2006; Yang and Tiersch, 2009b), and Guppy (*Poecilia reticulata*) (Sun et al., 2010) could be activated across a wide range of osmolalities with highest motility occurring in isotonic solutions for more than 72 h. Sperm activation of fishes from other viviparous families are rarely reported.

To date, the initiation of sperm motility has not been reported for the family Goodeidae (Cyprinodontiformes). These freshwater fishes are distributed throughout the Mexican Central Plateau (as viviparous species of the subfamily Goodinae) and the southwestern Great Basin of the United States (as oviparous species of the subfamily Empetrichthynae) (Webb et al., 2004). Goodeids are useful models to study palaeohydrological hypotheses regarding the Mexican Plateau, for which hydrological interpretation is difficult due to the complex history of volcanic and tectonic geological activity (Doadrio and Domínguez, 2004). Moreover, goodeids have been of great interest as models to study topics such as the evolution of viviparity (Helmstetter et al., 2016), phylogeography (Piller et al., 2015), and behavior (Sovrano et al., 2003). In addition to their research value, in the past 25 years, goodeids have become popular with aquarium hobbyists from Central and North America, and Europe (Maceda - Veiga et al., 2016). Unfortunately, the Goodeidae is considered as one of the most at-risk fish groups in the world (Duncan and Lockwood, 2001) due to heavily disturbed aquatic ecosystems in central Mexico resulting from water pollution, reductions in levels of ground and surface waters, basin deforestation, habitat destruction, and introduction of exotic species (Domínguez-Domínguez et al., 2006). The conservation status of Mexican goodeids includes 2 species categorized as extinct in the wild, 17 as critically endangered, 5 as endangered, 2 as threatened, and 11 as vulnerable and only 3 at lower risk rankings (Domínguez-Domínguez et al., 2005). Therefore, urgent action for goodeid conservation is needed, including restoration of natural habitats, reintroduction, captive breeding, and establishment of germplasm repositories via spermatogonia or sperm cryopreservation. An understanding of sperm activation of goodeids would provide the foundation for quality assessment and quality control in development of sperm cryopreservation protocols and germplasm repositories (Tiersch, 2011). In the present study we for the first time investigated the activation of sperm cells from goodeids as free sperm and as spermatozeugmata using Redtail Splitfin (*Xenotoca eiseni*) as a model. The specific objectives were to evaluate the effects of: (1) osmotic pressure and refrigerated storage (4

ºC) on activation of free sperm, (2) osmotic pressure, ions, and pH on dissociation of spermatozeugmata, and (3) $CaCl_2$ concentration and pH on sperm membrane integrity.

2. Materials and methods

2.1 Fish husbandry

Protocols for the use of animals in this study were reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee (Baton Rouge, LA, USA). The *X. eiseni* used in this study were bred by H. Grier (Uribe and Grier, 2010) from an aquarium population maintained by him, and transported at 6–12 months old from Florida to the Aquatic Germplasm and Genetic Resources Center (AGGRC) of the Louisiana State University Agricultural Center (Baton Rouge, LA). The fish were 2–3 years old prior to experiments. They were cultured at 20 – 26 ºC with 14 h:10 h (light:dark) photoperiod in four tanks of an 800-L recirculating aquaculture system and fed twice daily with tropical flakes (Pentair Aquatic Eco-systems, FL, USA) supplemented twice a week with thawed brine shrimp (Sally's Frozen Brine Shrimp , San Francisco Bay Brand, CA, USA). Additional water quality parameters were monitored weekly and held within acceptable ranges including: pH (7.0–8.0), ammonia (0–1.0 mg/L), and nitrites (0–0.8 mg/L).

2.2 Collection of free sperm and spermatozeugmata

Fish were anesthetized with 0.02% tricaine methanesulfonate (MS-222, Western Chemical, Inc. WA, USA) and standard body length and body wet weight were measured. Sperm cells of *X. eiseni* are concentrated in the anterior section of the testes. This section was dissected, rinsed, weighed, and placed in 100 uL of *Extender A* or *B* (Table 2) on a glass slide, and gently crushed with angled spade-tip forceps. Sperm cells were released into *Extender A* or *B* and collected with a pipette into a 1.5-mL centrifuge tube. Five microliters of each suspension collected from crushed testes were observed by use of a Makler® counting chamber composed of a base piece and a cover slip (Sefi-Medical Instruments, Haifa, Israel) and a dark-field (200-× magnification) microscope (CX41, Olympus Corporation, Tokyo, Japan). Experiments revealed discrete spermatozeugmata and sperm that were not associated with spermatozeugmata (referred as “free sperm”) (Fig. 1) in the suspension (referred as “mixed suspension” below) from every fish. The mixed suspensions were centrifuged (Marathon 13k/M, Fisher Scientific, PA, USA) for 10 s (at ~ 770× *g*) at room temperature (~ 24 ºC) followed by collection of supernatant as a free-sperm suspension and addition of 200 uL of *Extender A* or *B* to the pellet to prepare a spermatozeugmata suspension. Centrifugation at 600–1000× *g* had no effect on motility of sperm of *Xiphophorus species* (Dong et al., 2006b). Free-sperm suspensions with sperm motility higher than 10% were used in *Experiments 1* and *5* (about 20% of total males used), and spermatozeugmata suspensions were used in *Experiments 2*, *3*, and *4*. The number of spermatozeugmata in 100 squares of the counting chamber was adjusted to 10–30 by dilution. In this study, osmolalities were measured by use of a freezing point osmometer (Model 5010 OSMETTE III™, Precision Systems Inc., MA, USA) and pH of solutions was measured with pH meter (EcoSense® pH100A, YSI Inc., Ohio, USA). The pH of seminal fluid was estimated by placing several μL of crushed testis tissue on pH indicator strips (colorPHast pH 5.0–10.0, EM Science, New Jersey, USA), and the pH of the female reproductive tract was estimated

by inserting sections of pH strips (trimmed to ~ 1 mm × 20 mm) into the urogenital openings (three males and three females were used).

2.3 Sample preparation for scanning electron microscopy (SEM)

Images produced by SEM were used to illustrate morphologies differences of spermatozeugmata and free sperm. Spermatozeugmata from two males were pooled and suspended in primary fixative (1.6% paraformaldehyde and 2.5% glutaraldehyde in 50 mM cacodylate buffer) for 2–4 h at room temperature. The samples were washed in 100 mM cacodylate buffer, post-fixed in osmium tetroxide for 30 min, rinsed in water, and dehydrated through an ethanol series. Absolute (100%) ethanol was replaced by CO_2 in a standard critical point drier (Polaron E3000, Quorum Technologies, Lewes, UK), and the samples were dried at the critical point. The dried samples were mounted on 13-mm aluminum mount specimen stubs covered with carbon adhesive tabs, sealed with colloidal silver paste, coated with gold/palladium in a sputter coater (550X, Electron Microscopy Sciences, PA, USA) for 4 min to provide a coating thickness of 20–25 nm, and examined by use of an environmental scanning electron microscope (FEI Quanta 200, Thermo Fisher Scientific, MA, USA) using high vacuum mode at 20kV. Samples were processed and observed at the Core Microscopy Center of the School of Veterinary Medicine, Louisiana State University.

2.4 Evaluation of activation of free sperm and dissociation spermatozeugmata

To activate free sperm, suspensions were mixed with activation solutions (ratios described below and compositions described in Table 2) on the counting chamber and the coverslip was placed on top. The motility and curvilinear velocity (VCL) were measured at 10 s by use of a computer-assisted sperm analysis (CASA) system (HTM-CEROS, version 14 Build 013, Hamilton Thorne Biosciences, MA, USA). Based on previous testing, cell detection was set at a minimum of 25 pixels for contrast and 6 pixels for cell size: A total of 100 frames were captured in each measurement at 60 frames per 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. Two to three measurements from different viewing areas with 100–200 of sperm in each observation were applied and averaged for each observation. After capture, videos were reviewed and tracks with error readings (immotile sperm detected as motile by the CASA system) were eliminated manually.

To dissociate spermatozeugmata, suspensions were mixed with activation solutions on the base of the counting chamber and the coverslip was placed on top. Each of a total of 5–15 spermatozeugmata distributed across the viewing area were categorized into one of five phases by evaluating morphology of the spermatozeugmata and motion of sperm within the spermatozeugmata as Phase 0 (P_0) through Phase 4 (P_4), representing early through later dissociation stages. The frequencies of each dissociation phase (FDP) were referred as FDP_0 through FDP_4 and were used to evaluate dissociation of spermatozeugmata and motility capability of sperm within the spermatozeugmata (Liu et al., 2017). At P_0, the spermatozeugmata were quiescent; At P_1, sperm were activated within spermatozeugmata but remained associated; At P_2, the spermatozeugmata dissociated and sperm within them

swam away; At P_3, sperm stopped swimming away from the spermatozeugmata but remained vibrating in place; At P_4, all sperm movement ceased.

2.5 Experiment 1: Effect of osmotic pressure and refrigerated storage on activation of free sperm

Calcium-free HBSS was used as extender based on preliminary observations of activation effects of Ca^{2+}, and osmolality of 300 mOsmol/kg was based on preliminary measurement of blood plasma of 280–300 mOsmol/kg. Free-sperm suspension was mixed with *Activation Solution A* (Table 2) at ratios of 10:1, 6:1, 5:1, 4:1, 3:1, 2:1, and 1: 1 (suspension: *Activation Solution A*) to yield final osmolalities of 362, 398, 416, 440, 455, 516, 647 mOsmol/kg, or mixed with deionized water (pH ~ 7.0) at ratios of 4:1, 3:1, 2:1, 1:1, 2:3, 1:2, 1:3, 1:6, and 0:1 (water: suspension) to yield final osmolalities of 44, 58, 81, 135, 183, 209, 238, 275, and 305 mOsmol/kg. Motility and VCL was measured at 10 s after mixing. Because at 305 mOsmol/kg sperm had highest motility and VCL, free-sperm suspended in C-F HBSS305 were refrigerated at 4 ºC followed by motility and VCL measurements at 12, 24, 36, 48, 60, 72, 84, and 96 h. Sperm samples from five males were used in this experiment as separate replicates.

2.6 Experiment 2: Effect of osmotic pressure on spermatozeugmata dissociation

Electrolytic and non-electrolytic solutions were used to evaluate the effects of osmotic pressure on spermatozeugmata dissociation. Because preliminary observations showed possible effects of different ions on spermatozeugmata dissociation, thus, NaCl instead of HBSS were used. After mixing each of *Dissociation Solution A* or *B* (Table 2) with spermatozeugmata suspensions (*Dissociation Solution A* or *B*:spermatozeugmata suspension at 4:1 ratio), the FDP were recorded at 15 s, 10 min, 30 min, and 1, 3, 6, 12, 24, and 48 h. In this experiment, sperm samples from five males were used as replicates.

2.7 Experiment 3: Effect of ions on spermatozeugmata dissociation

Solutions of KCl, $CaCl_2$, $MgCl_2$, and $MnCl_2$ (*Dissociation Solution C*, Table 2) were mixed with the spermatozeugmata (*Dissociation Solution C*:spermatozeugmata suspension at 4:1), and the frequencies for each dissociation phase of spermatozeugmata were recorded at 15 s, 10 min, 30 min, and 1 h for KCl, $MgCl_2$, and $MnCl_2$, and 15 s, 10 min, 30 min, and 1, 3, 6, 12, 24, 48, 72, and 96 h for $CaCl_2$. The motility and VCL of free sperm released from spermatozeugmata were also measured from 10 min to 96 h. In this experiment, samples from five males were used as replicates.

2.8 Experiment 4: Effect of pH on spermatozeugmata dissociation

Based on preliminary observations, solutions with pH above 11.2 activated sperm within spermatozeugmata, and most of the sperm stopped moving within 5 min. Thus, two series of pH levels were used in this experiment as activation solutions (*Dissociation Solution D*, Table 2): (1) pH at 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0, and (2) pH at 11.2, 11.4, 11.6, 11.8, 12.0, 12.2, 12.4, and 12.6. After mixing activation media with the spermatozeugmata suspension (*Dissociation Solution D*:spermatozeugmata suspension at 4:1), FDP were recorded at 15 s, 1, 5, and, 10 min for pH-activation media (a), and at 15 s, 1

min, 2 min, and 5 min for pH-activation media (b). The motility and VCL of free sperm released from spermatozeugmata were recorded for pH-activation media (2). In this experiment, three replicates were used with two to three males pooled with equal volumes of spermatozeugmata suspensions in each replicate (for a total of three separate pools).

2.9 Experiment 5: Effect of pH and $CaCl_2$ concentration on membrane integrity of free sperm

Free-sperm suspensions (at an estimated concentration of 5×10^7 sperm cells per mL) were mixed with solutions of *Dissociation Solutions E* or *F* (*Dissociation Solutions E* or *F*:free-sperm suspension at 4:1) in 1.5-mL microcentrifuge tubes for 10 min, and 5 μL of this mixed suspension was diluted by mixing with 245 μL of NaCl solution at 300 mOsmol/kg. Three replicates with two males in each replicate were applied in this experiment.

Sperm plasma membrane integrity was determined using the fluorescent stains SYBR-14 and propidium iodide (PI) (PI/SYBR-14) (LIVE/DEAD® Sperm Viability Kit, cat. No. L-7011, Molecular Probes, OR, USA). The fluorescent dyes were prepared as described in Torres and Tiersch (2016) with the final concentration of SYBR 14 being 100 nM and of PI being 12 μM. Samples were incubated in the dark for 20 min at room temperature. Flow cytometry was performed with an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488-nm, 50 mW solid-state blue laser. Flow cytometer performance was assessed prior to the experiments using fluorescent validation beads (Spherotech, BD Accuri, BD Biosciences, CA, USA) to ensure that coefficient of variation values were less than 3.0% for the fluorescence detectors. Immediately before each test, the treated samples in centrifuge tubes were suspended with a vortex mixer, and 10,000 events (at 35 μL/min) were detected and gated by forward scatter (FSC) and right-angle side-light scatter (SSC) using CFlow Plus analysis software (version 264.21, BD Accuri, BD Biosciences, CA, USA). The FSC threshold was set at a default value of 80,000 to eliminate debris. Gating settings for the sperm population (gated events) used to exclude non-sperm events (particles) were based on our FSC and SSC profiles previously established for *X. eiseni* sperm. The PI fluorescence was detected by the FL3 detector (> 670 nm), and SYBR-14 was detected by the FL1 detector (533/30 nm). The regions representing the membrane-intact cell population (viable) and cells with compromised membranes (non-viable) were set manually. The total 10,000 events detected in each test were composed of gated cells (viable + non-viable) and debris. The percentage viable cells was calculated as (viable cells/ (viable cells + non-viable cells)) × 100%.

2.10 Data analysis

All statistical analyses were performed using SAS 9.4 (SAS Institute, NC, USA). One-way ANOVA with Tukey's multiple comparisons test were used to identify significant differences. Data were arcsine, root, or arcsine-square-root transformed prior to statistical analyses when assumptions of one-way ANOVA of normal distribution or equal variances were not met. When the assumptions were not met after transformation, Kruskal-Wallis non-parametric ANOVA (PROC NPAR1WAY) was performed. Simple linear regression (PROC REG) was used to evaluate the relationship between body wet weight and standard body length, and between body wet weight and testis weight. Quadratic regressions (PROC REG)

were performed to predict motility and velocity over osmolalities and times (quadratic models yielded higher r^2 than linear models based on preliminary tests). The results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Basic parameters and gamete collection

Males used in this study were mature with standard body lengths of 3.79 ± 0.45 cm (n = 30), body wet weights of 1.66 ± 0.61 g, and testis weights of 16.91 ± 9.06 mg. The body wet weight was positively ($P < 0.0001$) related to the standard body length with an $r^2 = 0.88$ and the testis weight was positively ($P < 0.0001$) related to the body wet weight with an $r^2 = 0.59$. The measured pH was 7.0–7.5 for the crushed testis and 8.0–8.5 for the female reproductive tract (measured at 5 – 15 mm from the urinogenital opening moving anteriorly towards the ovary). After gentle crushing of testes, free sperm and spermatozeugmata were each observed in the mixed suspensions. Spermatozeugmata remained at Phase 0 and Phase 1 and free sperm were quiescent or moving freely outside the spermatozeugmata (Fig. 2A). The motility of free sperm among individual males varied, ranging from 0 to 20%. Measurements estimated from the SEM images (Fig. 1) showed spermatozeugmata from *X. eiseni* were nearly spherical with observed diameters of 30–35 μm. Surfaces of spermatozeugmata were composed of intermingled flagella with only a few (< 10 in each spermatozeugmatum) visible sperm heads. The free sperm (Fig. 1) had a slightly elongated head (observed sizes ~ 1.5 × 2 μm), an annular midpiece (~ 0.5 × 1 μm) at the bottom of heads, and a flagellum (~ 27 μm long and 0.25 μm wide). These measurements may be influenced due to SEM fixation (Dong et al., 2006a), but overt artifacts were not detected.

3.2 Effect of osmotic pressure and refrigerated storage on activation of free sperm

There were significant differences for motility and VCL of free sperm across osmolality ($P < 0.0001$, $P = 0.0011$) from 44–647 mOsmol/kg, and time ($P < 0.0001$, $P = 0.0283$) from 0–96 h. Motility was inhibited (< 1%) in C-F HBSS at osmolalities of < 81 and > 516 mOsmol/kg (Fig. 3), and was at peak at 305 mOsmol/kg. The VCL increased with osmolality, remained at between 85.4 ± 13.6 and 95.6 ± 5.6 μm/s for183 to 398 mOsmol/kg, and dropped to the lowest points (52.0 ± 3.1 μm/s) the osmolality was raised to 516 mOsmol/kg. Regressions showed significant quadratic relationships between osmolality and motility ($P < 0.0001$, $r^2 = 0.78$), and VCL ($P < 0.0001$, $r^2 = 0.97$).

Free sperm had highest motility (20 ± 8%) after15 s in C-F HBSS305 (Fig. 3). During storage at 4 ºC, the motility continuously decreased to 6 ± 3% at 48 h and 2 ± 1% at 84 h. No free sperm were observed moving after 96 h. The VCL remained stable from 15 s to 24 h but declined to 79.3 ± 32.8 μm/s at 36 h. Regressions showed significant quadratic relationships between time and motility ($P < 0.0001$, $r^2 = 0.96$) and VCL ($P = 0.0152$, $r^2 = 0.81$).

3.3 Effect of osmotic pressure on spermatozeugmata dissociation

Solutions of NaCl and glucose at osmolalities of 50 to 800 mOsmol/kg did not dissociate spermatozeugmata to Phases 2, 3, or 4, thus only FDP_1 was recorded (Table 1). At 15 s, all

spermatozeugmata were at Phase 0. At 10 min, NaCl from 300 to 600 mOsmol/kg dissociated spermatozeugmata with the highest FDP_1 (28 ± 8%) at 300 mOsmol/kg. At 1 h, spermatozeugmata were dissociated to Phase 1 across the widest range in NaCl (from 50 to 600 mOsmol/kg), and in glucose at 400 and 500 mOsmol/kg. At 24 h, Phase-1 spermatozeugmata were only observed at 300 mOsmol/kg in NaCl and glucose. After 48 h, no active spermatozeugmata were observed in NaCl or glucose solutions.

3.4 Effect of ions on spermatozeugmata dissociation

No spermatozeugmatum were observed at Phases 2, 3, or 4 when suspended in KCl, $MgCl_2$, and $MnCl_2$ within 1 h, and only FDP_1 was observed. At 15 s and 10 min, concentration had no effects on FDP_1 of bundles mixed with KCl, $MgCl_2$, and $MnCl_2$ (Fig. 4). At 10 min, spermatozeugmata began to be dissociated in KCl with FDP_1 from 6 ± 4 % at 5 mM to 11 ± 5% at 160 mM. At 30 min and 1 h, FDP_1 increased significantly ($P < 0.03$) with concentrations of KCl, $MgCl_2$, and $MnCl_2$ except for $MnCl_2$ at 1 h). From 5 s to 1 h, FDP_1 for had significant differences between 5 mM (a physiological concentration) of KCl, $MgCl_2$, and $MnCl_2$ ($P < 0.001$, generally $MnCl_2 > MgCl_2 > KCl$). In general, FDP_1 increased with time in all three solutions.

The spermatozeugmata were activated into Phase 2 by $CaCl_2$, and therefore FDP_2 from 15 s to 24 h were reported (Fig. 5A) (after 24 h, spermatozeugmata concentrations dropped and there were too few spermatozeugmata in viewing areas to calculate an FDP). At 15 s, no spermatozeugmata were at Phases 2, 3, or 4. At 10 min, and FDP_2 increased from 1 ± 2% at 5 mM $CaCl_2$ to 25 ± 6% at 160 mM $CaCl_2$. After 30 min, FDP_2 in all concentrations of $CaCl_2$ increased to a highest value before decline. Motile sperm were observed in the spermatozeugmata suspensions when mixed with $CaCl_2$ (Fig. 2B). The highest motility was at 30 min for 10 and 80 mM $CaCl_2$, at 1 h for 5, 20, and 160 mM $CaCl_2$, and at 3 h for at 40 mM $CaCl_2$ (Fig. 5B). At 24 h, the motility was between 3 ± 2 and 7 ± 4% in all concentrations and at 96 h no motility higher than 1% was observed. The VCL of sperm activated in 160 mM $CaCl_2$ remained between 70.9 ± 10.9 and 76.8 ± 5.1 μm/s from 10 min to 6 h, and decreased to 58.7 ± 4.3 μm/s at 12 h, and 48.6 ± 11 μm/s at 24 h. Regressions showed significant quadratic relationships between time and VCL ($P = 0.0123$, $r^2 = 0.89$). The VCL values for $CaCl_2$ at concentrations below 160 mM and for 160 mM after 24 h were not recorded because motilities ≤ 10% can result in inaccurate measurements by CASA.

3.5 Effect of pH on spermatozeugmata dissociation

Spermatozeugmata remained at Phase 0 for 10 min after mixing with NaCl-HCl or NaCl-NaOH solutions with pH at 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0. In NaCl-NaOH at pH 12.6, spermatozeugmata disintegrated within 2 s without sperm movement.

For pH from 11.2 to 12.4 (Fig. 6), NaCl-NaOH solution at pH 11.2 and 11.4 activated spermatozeugmata into Phase 1 from 15 s to 5 min. At 15 s, spermatozeugmata were activated to Phase 2 with pH 11.6 to 12.4 (Fig. 2C) (100% at Phase 2 at pH 11.8 and 12.0). At pH 12.4, 62 ± 8% of spermatozeugmata were activated into Phase 4 within 1 min. At 1 min, FDP_2 increased at pH 11.6, but decreased at pH 11.8, 12.0, 12.2, and 12.4 compared to those at 15 s and all sperm cells stopped movement at pH 12.4. The FDP_3 at pH 11.8, 12.0,

and 12.2 continuously increased from 15 s to 5 min. At 5 min, no spermatozeugmata at Phase 2 was observed.

Free sperm released from the spermatozeugmata were observed by used of CASA system. At 15 s, the motility (Fig. 7) of sperm released from spermatozeugmata increased significantly ($P = 0.0004$) with pH. The VCL of sperm increased significantly ($P = 0.0003$) with pH. From 15 s to 2 min, the motility and velocity at all pH levels continuously decreased. At 2 min the motility declined to 14 ± 8% at pH 11.8 and < 3% at all other pH levels. At 5 min, no motility was observed at any pH levels.

3.6 Effect of pH and $CaCl_2$ concentration on membrane integrity of free sperm

No significant differences were observed for the percentage of viable cells ($P = 0.2033$) among different concentrations of $CaCl_2$ (Fig. 8). Among different pH levels, the percentage of viable cells at pH 12.5 was lower ($P < 0.0001$) than those at pH from 7.0 to 12.0.

4. Discussion

During collection of male gametes by gentle crushing of testes from *X. eiseni*, we found the majority of spermatozeugmata remained un-dissociated although motile free sperm were present in some individuals. Interestingly, spermatozeugmata from poeciliids, another internally fertilized freshwater group in Cyprinodontiformes, respond differently from those of goodeids when physical pressure is applied to the testes. For example, most of sperm from Guppy and *Xiphophorus* species can be released from spermatozeugmata by application of gentle pressure on the testes during sperm collection (Sun et al., 2010; Yang et al., 2006). The SEM images revealed that the midpieces of sperm of *X. eiseni* were not longitudinally elongated as observed in *Xiphophorus* species (Huang et al., 2004c), and surfaces of bundles were composed with sperm tails rather than heads as observed in *Xiphophorus* species (Grier et al., 1978). These different morphological structures could make bundles of *X. eiseni* more difficult to be dissociated. Further studies are needed on the effects on chemical environment and physical contraction within the female reproductive tract on dissociation of spermatozeugmata.

The testis weight of *X. eiseni* used in this study was 16.91 ± 9.06 mg, and only the anterior half of the testes were filled with sperm or spermatozeugmata, which may contain estimated 8.45 μL of seminal fluid on average based on the assumption proposed in studies of *Xiphophorus* species (Huang et al., 2004a). The small volume of sperm that can be collected from individual fish may require the pooling of testis samples for experiments or repository activities. The significant correlation between body wet weight and testis weight can be useful to estimate the number of fish needed for such activities (Yang and Tiersch, 2009b). The osmolality and ion concentrations of the internal testis environment were not measured in this study due to the miniscule volumes available. Sperm from Banded Houndshark (Minamikawa and Morisawa, 1996) and Rainbow Trout (Morisawa and Morisawa, 1986) can acquire the capacity for movement during their passage from the testis along the sperm duct, however because we collected male gametes directly from testes, we were unable to identify whether there was a motility acquisition effect during transport of spermatozeugmata in the male reproductive tract.

Upon arrival at the female, spermatozeugmata must be dissociated and the sperm activated to transit the reproductive tract (Fig. 9). Viviparous species employ internal fertilization, releasing sperm into the female reproductive tract, which is usually isotonic to the body and intracellular environment of sperm, and therefore the isotonic environment appears to be able to activate sperm motility (Tiersch, 2011). *Experiment 1* showed motility of free sperm was induced across a wide range of osmolalities with highest motility at 305 mOsmol/kg, and was inhibited in hypotonic and hypertonic solutions. This pattern is consistent with studies on poeciliids, such as Guppy (Sun et al., 2010), Mosquitofish (Morisawa and Suzuki, 1980), and *Xiphophorus* species (Huang et al., 2004a; Huang et al., 2004b), in which highest motilities were observed near 250–350 mOsmol/kg. The highest motility observed in the present study was about 25%, lower than those observed in poeciliids (70–90%) (Huang et al., 2004b; Huang et al., 2009). The relatively low motility might result from seasonal variations of sperm motility. For example, our unpublished observations after the present study found during the natural spawning season (usually from February to June) more than 50% of males had free sperm with motility > 10% (the highest motility observed was 80%), whereas from September to December more than 80% of males had immotile (< 1%) free sperm. The optimum VCL of *X. eiseni* at isotonic conditions was around 85 to 95 μm/s, which was faster than that reported for Guppy (42.66 to 83.95 μm/s) (Boschetto et al., 2011; Locatello et al., 2006) and *Xiphophorus nigrensis* (31.5 μm/s) (Smith and Ryan, 2010). In addition to sperm motility, velocity may be essential for competitive fertilization success among males for internally fertilized species (Boschetto et al., 2011; Gasparini et al., 2010). However, in the quality evaluation of sperm cryopreservation of internally fertilized fishes, velocity has been rarely reported. The results of *Experiment 1* suggest that after free sperm are dissociated from spermatozeugmata by crushing of the testes, they are capable of travel in isotonic conditions similar to the female reproductive tract.

What about the spermatozeugmata that are unable to be dissociated by physical pressure? Could dissociation be induced by certain physiochemical conditions in the female? In *Experiment 2*, NaCl and glucose solutions dissociated the spermatozeugmata into Phase 1 for 24 h and the dissociation was inhibited in hypotonic and hypertonic conditions. This osmolality-dependent pattern is compatible with the activation of free sperm observed in *Experiment 1*, reconfirming the effect of osmotic pressure on sperm activation and duration of sperm movement. However, NaCl and glucose was not sufficient to dissociate spermatozeugmata into advanced phases (activated sperm only vibrated within the spermatozeugmata), suggesting that isotonic osmotic pressure itself within the female reproductive tract may not be sufficient to release sperm from the spermatozeugmata.

In addition to osmotic pressure, ions (especially Na^+, K^+, Mg^{2+}, and Ca^{2+}) were important in sperm activation due to their function of mediating cell signaling or changing cell membrane potential (Alavi and Cosson, 2006). In *Experiment 3*, solutions with different concentrations of K^+, Mg^{2+}, and Mn^{2+} activated sperm within spermatozeugmata but did not dissociate the spermatozeugmata into Phase 2. Solutions of $CaCl_2$ dissociated spermatozeugmata into Phase 2 and activated free sperm with a concentration-dependent manner. The different effects of ions on spermatozeugmata dissociation after 1 h might result from their different capabilities for signaling intracellular activities or altering the plasma membrane potential. In salmonids, a relatively higher concentration of extracellular

K^+ suppresses sperm motility by depolarizing sperm cells, but bivalent cations such as Ca^{2+} and Mg^{2+} can counter the inhibitory action of K^+ (Alavi and Cosson, 2006). This is consistent with the results from the present study with dissociation effect of ions estimated as: $Ca^{2+} > Mn^{2+} > Mg^{2+} > Na^+ > K^+$. It has been shown that influxes of Ca^{2+}, and the ensuing signaling initiates sperm motility of Common Carp (*Cyprinus carpio*) (Krasznai et al., 2000). Further studies are needed on whether Ca^{2+} influx triggers activation of sperm within bundles of *X. eiseni*. In another hypothesis, ions could function extracellularly on dissociation of spermatozeugmata for internally fertilized fish. For example, in dissociation of sperm bundles from Guppy, no Ca^{2+} influx was observed during dissociation and the effect rank of cations on dissociation ($Li^+ < K^+ < Cs^+ < NH_4^- < Co_2+ < Mg_2+ < Ba_2+ < Ca_2+ < Mn_2+$) correlated to the ability to weaken hydrophobic bonds of extracellular proteins (Tanaka and Oka, 2005). This hypothesis might also account for the different effects of ions on dissociation of spermatozeugmata in the present study.

After sperm are released from spermatozeugmata inside the female, they must travel through the reproductive tract toward the ovary to fertilize oocytes (Fig. 9). This process requires sperm from internally fertilized fish to possess a longer swimming duration than needed for externally fertilized species. For example, sperm remained motile for longer than 2 d in Guppy (Sun et al., 2010) and 10 d in Green Swordtail when held in HBSS (Huang et al., 2004b). This is in contrast to most externally fertilized species such as Zebrafish (*Danio rerio*), which sperm remain motile for < 10 min (Yang et al., 2007b). But it is consistent with our results that free sperm collected by crushing of testes (*Experiment 1*), and released from dissociation of spermatozeugmata by $CaCl_2$ (*Experiment 3*) could remain motile for longer than 3 d. It has been suggested that for *Xiphophorus* species the narrow cylindrical head structures and well-developed mitochondrial sheaths in the elongated midpiece of sperm cells contribute to the long motility duration (Huang et al., 2004c; Yang et al., 2007b), however these structures were not present in sperm from *X. eiseni*.

Another relevant question is whether sperm really need 2–3 d of continuous swimming within the female prior to fertilization? The length of the female reproductive tract (from the urinogenital opening to the anterior end of the ovaries) of mature goodeids is usually about 5–15 mm (our unpublished measurements). If we calculate the transit time to swim this direct length with the free sperm velocity observed in *Experiment 1* (95 μm/s at 305 mOsmol/kg), sperm would require a minimum of 53–158 s to complete the distance. Because the resistant forces (such as fluid viscosity) and pathways inside the reproductive tract are unknown and cannot be included in the calculation, the actual time sperm spend in traveling through the tract could be different from this estimation. It was suggested that Guppy sperm cells require about 15 min to migrate to the ovarian cavity after they were dissociated from spermatozeugmata (Skinner and Watt, 2007). Upon arrival at the ovary, sperm may need to continue swimming to contact oocytes to achieve fertilization (Fig. 9). However, oocytes may not always be ready to be fertilized. This is not a problem for species that can store sperm within the ovary or reproductive tract for months prior to fertilization such as poeciliids (Potter and Kramer, 2000; Uribe et al., 2016). But for goodeids, which are believed to not store sperm (Garcia et al., 1994), the longer swimming duration may be important for providing extra time for synchronization of fertilization events.

In addition to osmotic pressure and ions, the pH of an activation solution usually affects motility, but to a low extent (Alavi and Cosson, 2005). In *Experiment 4* NaCl-HCl or NaCl-NaOH solutions at pH of 2.0 to 11.0 did not dissociate spermatozeugmata; pH from 11.2 to 11.4 could activate sperm within spermatozeugmata but failed to dissociate them into Phase 2; but pH from 11.6 to 12.4 dissociated spermatozeugmata into Phase 2 and activated released free sperm within a short time (1 min) with velocity higher than sperm activated with HBSS305 and $CaCl_2$. To our knowledge this is the first report that sperm from a freshwater fish could be activated by such alkaline solutions. Other studies have shown that the optimum sperm motility in Rainbow Trout was at pH 9.0 (when temperatures were between 5 and 21°C) (Alavi and Cosson, 2005). In Common Carp, sperm motility was pH independent between pH 6.04 and 9.03 (Redondo-Müller et al., 1991). The motility and duration of sperm from Paddlefish were more sensitive to pH. For example, Paddlefish sperm had 90–95% motility at pH 8.0 at 60 s after activation, however the motility was 80–85% at pH 7.0, and 60–65% at pH 6.0 (Linhart et al., 2002). For viviparous species, Guppy sperm was found to tolerate a wide range of pH ranging from 5.6 to 7.8, but motility was affected negatively by pH > 7.8 (Sun et al., 2010).

It is interesting and unclear why spermatozeugmata in *Experiment 4* can be activated at such high pH levels. One possibility was that extracellular pH affected the intracellular proton concentration, which subsequently affected the membrane potential, as well as motility behavior (Boitano and Omoto, 1991). Another interpretation could be that high pH levels dissolved presumptive substances that bind sperm within spermatozeugmata, such as proteins (Takemura et al., 1999) or polysaccharides (Sasikala and Subramoniam, 1987). In addition, calcium channels might also be involved in high-pH induced activation of spermatozeugmata. For example, in mammals, which are also internally fertilized vertebrates, intracellular alkalinization potentiates the current of CatSper, a calcium channel, to increase intracellular calcium as key signaling to induce sperm hyperactivation (Kirichok et al., 2006). Further studies are needed on pathways by which intracellular pH and calcium signaling (and their interactions) initiates activation of sperm or dissociation of spermatozeugmata from internally fertilized fish, as well development of methodologies to study these signaling pathways in spermatozeugmata.

In *Experiments 3 and 4*, spermatozeugmata were dissociated and sperm were activated when mixed with activation media with $CaCl_2$ at 5–160 mM and NaCl-NaOH at pH 11.6–12.2. However, it is not likely in nature that the testis or female reproductive tract have such a high pH or concentration of Ca^{2+}. For example, Ca^{2+} concentrations of seminal plasma were below 5 mM for Salmonidae, Acipenseridae, and marine fishes, and below 50 mM for Cyprinidae. Values for pH were at 7.1–8.2 for sperm and seminal plasma of Rainbow Trout (Alavi and Cosson, 2006), and 8.0–8.5 within the female reproductive tracts of *X. eiseni* as estimated in the present study. In addition, the high values of Ca^{2+} and pH could have toxic effects on sperm viability. Interestingly, in *Experiment 5* we found that $CaCl_2$ concentrations at 0–150 mM and pH at 7.0–12.0 had no significant effect on membrane integrity of free sperm from *X. eiseni* after activation for 10 min, whereas pH at 12.5 had a damaging effect on membranes as assessed by flow cytometry. It is unclear how sperm can remain intact and activate at these high pH levels (pH 10.0–12.0). However, the motility duration of sperm activated at pH of greater than 11.0 was less than 5 min, shorter than the 3 d for free sperm

when activated at lower pH, suggesting that there was a negative effect on viability at high pH that was not identified by flow cytometry. The high levels of pH and calcium we applied in the present study may not occur in the female reproductive tract or ovary of *X. eiseni*, however, dissociation of spermatozeugmata with $CaCl_2$ (50–150 mM) and high-pH solution (11.6–12.2) can be applied experimentally as a method to evaluate spermatozeugmata quality. For example, in studies of cryopreservation, the dissociation of thawed spermatozeugmata with NaCl-NaOH at pH 11.8 could be used as an indicator of sperm viability. The actual values for, and interactions of pH and Ca^{2+} in seminal plasma and those within the female reproductive tract should be further investigated.

5. Conclusions

Viviparity and internal fertilization among fishes has evolved independently and sporadically and the activation mechanisms of sperm and dissociation spermatozeugmata among different taxa are poorly understood. In the present study, we for the first time report the activation of male gametes from goodeids and the different features between activation of free sperm and dissociation of spermatozeugmata from an internally fertilized fish. Our study of activation factors such as physical contraction, osmotic pressure, ion concentration, and pH could provide a basis to study activation of male gametes from other internally fertilized species. Osmolalities at about 300 mOsmol/kg can be used for extenders, and solutions for activation of free sperm, and dissociation of spermatozeugmata of goodeids. Calcium can be considered in research or applied practices to dissociate sperm bundles. We for the first time reported the activation of sperm from a freshwater fish at pH above 11.4. Although the mechanism is not understood, this activation method may be applied as an indicator of sperm viability for research and quality control efforts. Further study is needed on the signaling pathways by which osmotic pressure, ions and pH (and their interactions) activate sperm and spermatozeugmata from livebearing fishes. Conservation efforts for endangered goodeids made by scientists and hobbyists, include captive maintenance, and long-term projects to rehabilitate habitats, allowing goodeids to persist or be re-introduced into the wild (Domínguez-Domínguez et al., 2005). Cryopreservation of sperm is an important tool for conservation and recovery of threatened populations (Howard et al., 2015). Understanding dissociation of spermatozeugmata and sperm activation are essential for evaluation of sperm quality and artificial insemination in development of sperm cryopreservation protocols. The present study can assist the development of germplasm repositories of imperiled goodeids, and future work should include standardization of quality evaluation of sperm bundles, protocol establishment of sperm cryopreservation, and generalized strategy development for conservation of livebearing fishes assisted by repositories of cryopreserved sperm.

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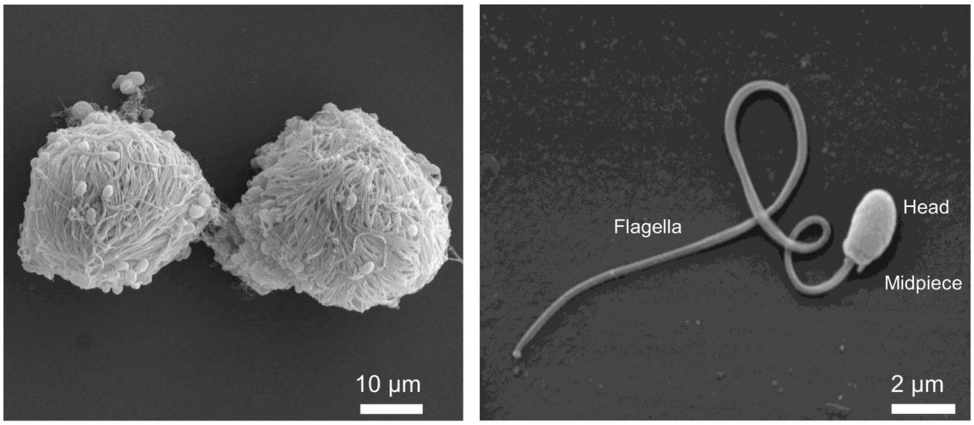

Fig. 1.

Representative scanning electron micrographs of spermatozeugmata (left) and free sperm (right) collected by gentle crushing of testis of *X. eiseni*.

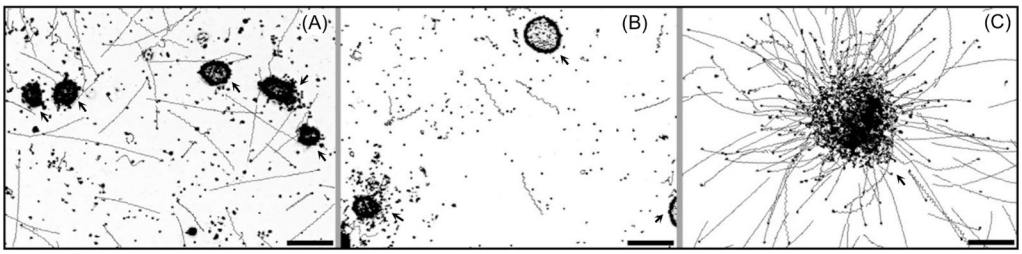

Fig. 2.

Activation of free sperm and dissociation of spermatozeugmata from *X. eiseni* observed by use of a computer-assisted sperm analysis system at 200-× magnification (100 frames during 1.67 s). The dots in images indicate quiescent sperm and tracks indicate sperm movement. Black arrows indicate spermatozeugmata in different phases. The length of black bars (lower right) represents 100 μm. Colors of original images were reversed and converted into grey scale. (A) Sperm suspension in Ca^{2+}-free HBSS305 at 1 h after collection. Spermatozeugmata were at Phase 1 with shaking sperm attached. Free-swimming sperm and quiescent sperm were observed outside spermatozeugmata. (B) Spermatozeugmata suspension at 30 min after activation by 160 mM $CaCl_2$. Spermatozeugmata were at Phase 0 and Phase 1. Moving and quiescent sperm were observed outside spermatozeugmata. (C) Spermatozeugmata at Phase 2 at 15 s after activation by NaCl-NaOH solution at pH 11.8. Sperm were actively moving away from the spermatozeugmata.

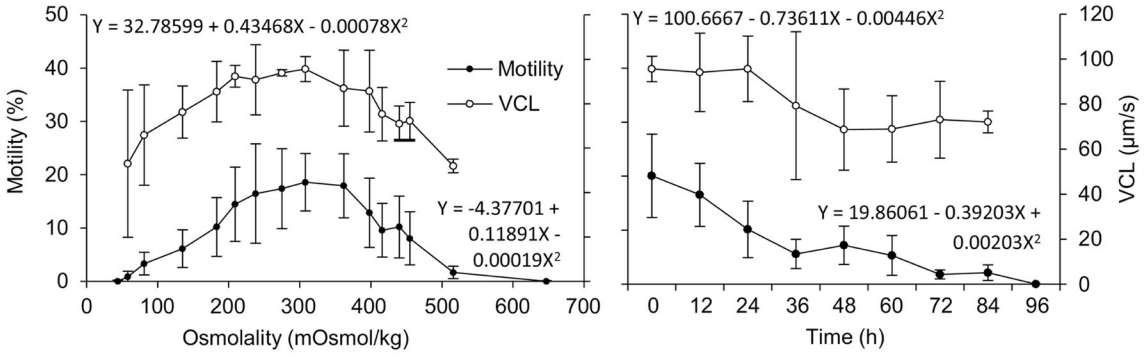

Fig. 3.

Motility and velocity of free sperm from *X. eiseni* at (left) different osmolalities and (right) at 305 mOsmol/kg during storage at 4 ºC for 96 h. The 0 h indicates the observation at 15 s. Data are presented as mean ± S.D. of five males. Equations were produced by quadratic regressions.

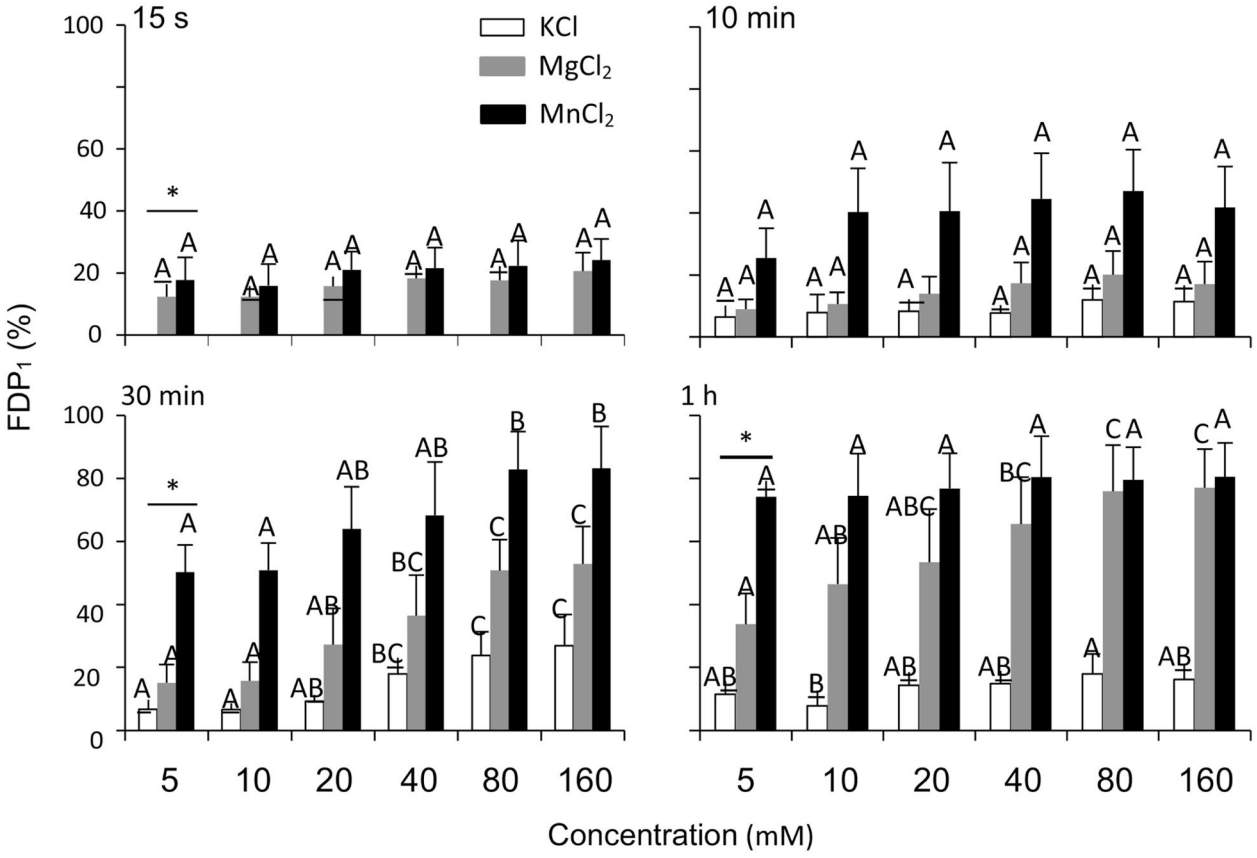

Fig. 4.

The percentage of spermatozeugmata at Phase 1 from *X. eiseni* after activation by KCl, $MgCl_2$, and $MnCl_2$ at different concentrations. Different concentrations of each activation solution sharing the same letter were not significantly different. Asterisks above black lines indicate significant differences among three activation solutions at 5 mM.

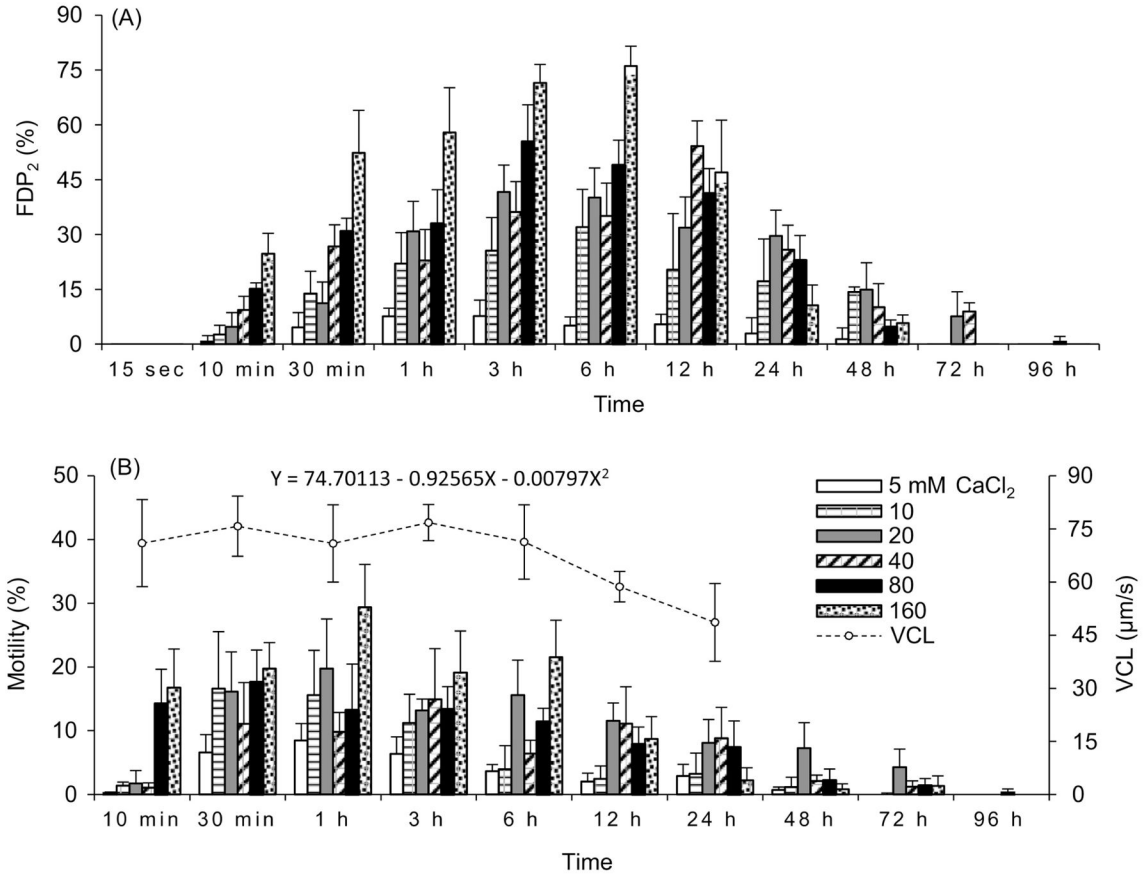

Fig. 5.

The activation of spermatozeugmata from *X. eiseni* by different concentration of $CaCl_2$. (A) Frequencies of spermatozeugmata at Phase 2 during 24 h after activation. (B) The motility and velocity of sperm released from spermatozeugmata after activation by $CaCl_2$ at 160 mM during 96 h stored at 4ºC. Data were presented as mean ± S.D. of five males. Equations were produced by quadratic regressions.

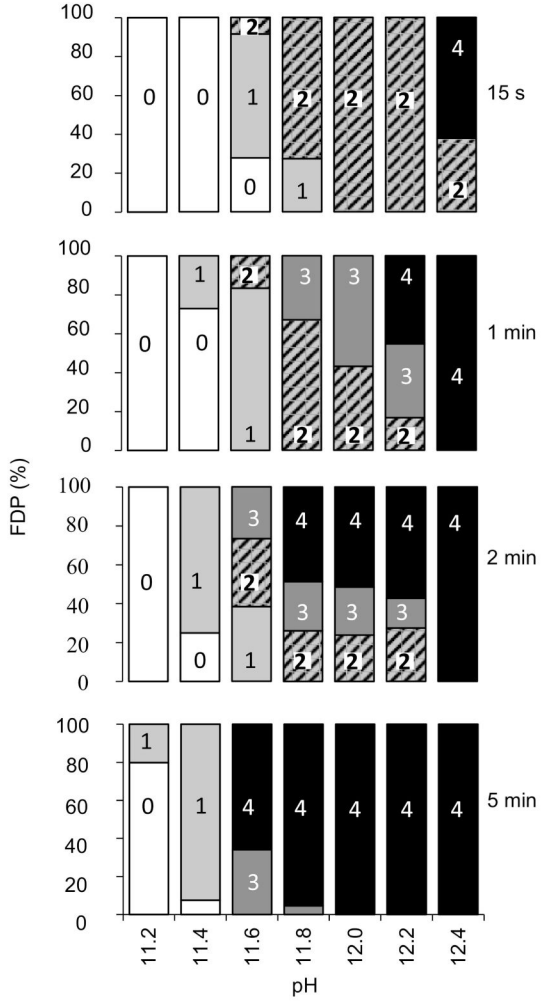

Fig. 6.

Frequencies of phases of spermatozeugmata of *X. eiseni* activated by NaCl-NaOH solutions with pH from 11.2 to 12.4 at 15 s, 1 min, 2 min, and 5 min. Numbers (0–4) on bars indicate Phases 0 to 4.

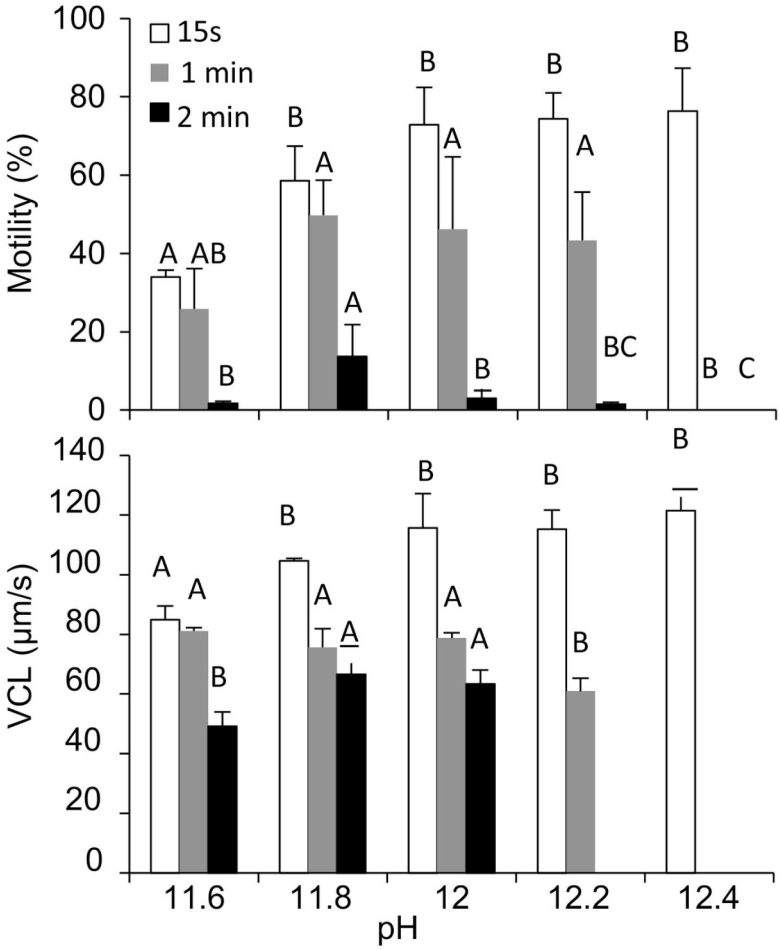

Fig. 7.

The motility (top) and velocity (bottom) of sperm released from spermatozeugmata after activation at different pH at 15 s (white circles and bars), 1 min (grey circles and bars), and 2 min (black circles and bars). Data were presented as mean ± S.D. of three replicates with two to three males in each replicate. Different pH of each time sharing the same letter were not significantly different.

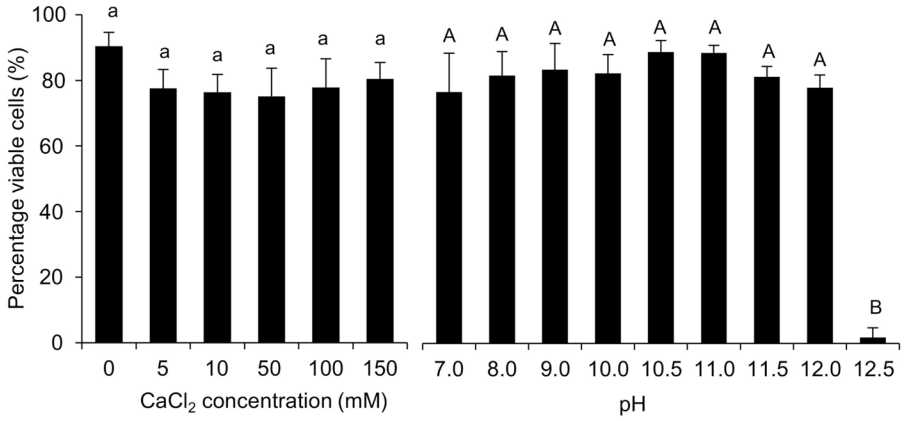

Fig. 8.

Percent of cells with intact membranes analyzed by flow cytometry after activation by different concentrations of $CaCl_2$ and NaCl at different pH. Treatments sharing the same letter (lower case letters for different concentrations of $CaCl_2$ and capitalized letters for different pH) were not significantly different. Data were presented as mean ± S.D. of three replicates with two males in each replicate.

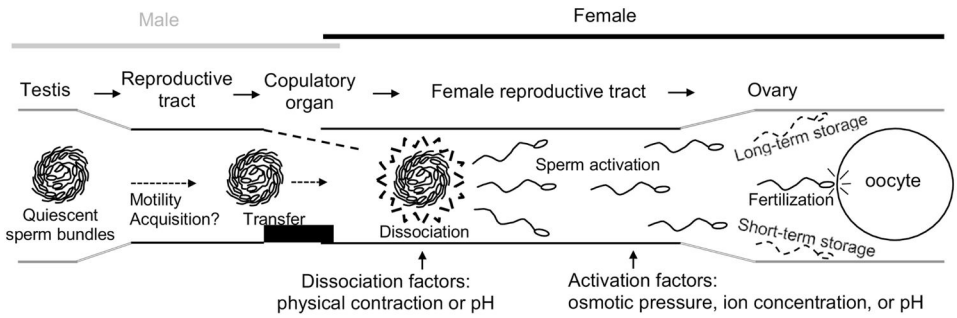

Fig. 9.

Schematic of the major steps involved in the journey of male gametes from testis to ovary of internally fertilized fish in the Cyprinodontiformes. Sperm bundles are un-dissociated and sperm are immotile in testes. It is unclear whether sperm within bundles acquire motility potential during their passage through the male reproductive tract. Bundles are transferred into females by copulatory organs, such as gonopodia (the intromittent type) of poeciliids or andropodia (the non-intromittent type) of goodeids. Upon the arrival within the female reproductive tract, the dissociation of bundles may result from physical contraction, or dissolution of presumptive substances that bind sperm within bundles by elevated pH. The dissociation could also be triggered by activation of sperm within bundles. Activation of sperm can be affected by osmotic pressure, ion concentration or pH. After activation, free sperm need to travel through the tract before fertilizing oocytes in the ovary. However, sometimes there are no available oocytes to fertilize immediately. Thus, before fertilization free sperm may persist for a short term (several d) as in goodeids or long term (several months) as in poeciliids. The long motility duration (several d) of sperm from internally fertilized species is likely required for efficient transit and fertilization.

Table 1

The frequencies (mean ± S.D.) of Phase-1 spermatozeugmata from *X. eiseni* after activation with NaCl and glucose solutions at different osmolalities (n = 5).

Osmolality (mOsmol/kg)	Frequency of Phase-1 spermatozeugmata (%)								
	15 s	10 min	30 min	1 h	3 h	6 h	12 h	24 h	48 h
NaCl solutions									
50	0	0	0	2 ± 4	0	0	0	0	0
100	0	0	0	6 ± 13	0	0	0	0	0
200	0	0	14 ± 17	14 ± 5	2 ± 4	8 ± 4	0	0	0
300	0	28 ± 8	10 ± 7	26 ± 13	6 ± 9	6 ± 5	16 ± 9	8 ± 8	0
400	0	12 ± 4	14 ± 5	24 ± 11	8 ± 8	8 ± 8	0	0	0
500	0	14 ± 5	22 ± 19	2 ± 4	8 ± 4	6 ± 9	0	0	0
600	0	12 ± 4	14 ± 11	2 ± 4	0	0	0	0	0
700	0	0	0	0	0	0	0	0	0
800	0	0	0	0	0	0	0	0	0
Glucose solutions									
50	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	0	0
300	0	0	0	0	2 ± 4	2 ± 4	2 ± 4	10 ± 10	0
400	0	0	0	8 ± 8	6 ± 5	4 ± 9	0	0	0
500	0	0	0	4 ± 5	4 ± 5	4 ± 5	14 ± 13	0	0
600	0	0	0	0	2 ± 4	4 ± 5	4 ± 9	0	0
700	0	0	0	0	0	4 ± 5	0	0	0
800	0	0	0	0	0	0	0	0	0

Comp Biochem Physiol A Mol Integr Physiol. Author manuscript; available in PMC 2019 April 01.

Table 2

Description of solutions used in the present study.

Solution name	Experiment	Description	Osmolality (mOsmol/kg) *	pH **
Extender A	*Experiment 1*	Calcium-free HBSS300 (C-F HBSS300) ***	300 ± 5	7.2
Extender B	*Experiments2, 3, 4,* and *5*	145 mM NaCl ****	300 ± 5	7.0
Activation Solution A	*Experiment 1*	Calcium-free HBSS at 1,000 mOsmol/kg (C-F HBSS1,000) ***	1,000 ± 10	7.2
Dissociation Solution A	*Experiment 2*	Adjustment of osmolalities of formulation for 485 mM NaCl	50, 100, 200, 300, ..., and 800	8.0
Dissociation Solution B	*Experiment 2*	Adjustment of osmolalities of formulation for 945 mM glucose	50, 100, 200, 300, ..., and 800	8.0
Dissociation Solution C	*Experiment 3*	KCl, CaCl2, MgCl2, and MnCl2 at concentrations of 5, 10, 20, 40, 80, and 160 mM ****	300 ± 30	8.0
Dissociation Solution D	*Experiment 4*	Mixture of 145 mM NaCl solution with NaOH (for pH ≥ 7) or HCl (for pH < 6).	300 ± 10	as indicated
Dissociation Solution E	*Experiment 5*	CaCl2 solutions at concentrations of 0, 5, 10, 50, 100, and 150 mM *****	300 ± 30	8.0
Dissociation Solution F	*Experiment 5*	145 mM NaCl solutions at pH of 7.0, 8.0, 9.0, 10.0, 10.5, 11.0, 11.5, 12.0, and 12.5 adjusted by addition of NaOH	300 ± 10	as indicated

* Osmolalities were obtained by adjustment of the water volumes (*Extender A* and *B*, *Activation A*, and Dissociation *A*, *B*, *D*, and *F*), or addition of NaCl (*Dissociation D* and *E*).

** The pH of 8.0 was obtained by addition of 20 mM Tris-HCl at pH 8.0. The pH of 7.0 to 7.2 were used for extenders because pH of the testes was measured as 7.0–7.5 in the present study, whereas pH 8.0 was used for activation solutions (except for HBSS solutions) because pH of the female reproductive tract was measured as 8.0–8.5.

*** The C-F HBSS solutions were prepared by adjusting the water volumes within the formulation of C-F HBSS300 (137 mM NaCl, 5.4 mM KCl, 1.0 mM $MgSO_4$, 0.25 mM 145, Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.5 mM glucose (Yang et al., 2007a).

**** NaCl at concentrations of 140, 135, 120, 95, and 40 mM was added to 5, 10, 20, 40, and 80 mM of KCl, $CaCl_2$, $MgCl_2$, and $MnCl_2$ to produce a constant osmotic pressure of 300 ± 30 mOsmol/kg. The osmolalities of 160 mM of KCl, $CaCl_2$, $MgCl_2$, and $MnCl_2$ were already > 300 mOsmol/kg (358, 476, 476, and 470 mOsmol/kg), thus no NaCl was added.

***** In 0, 5, 10, and 50 mM of $CaCl_2$, 145, 140, 135, and 75 mM of NaCl were added to yield a final osmolality of 300 ± 30 mOsmol/kg. No NaCl was added to 100 mM (300 ± 10 mOsmol/kg) or 150 mM $CaCl_2$ (412 mOsmol/kg).