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Osmotic activation of sperm motility via water flow through aquaporins in the freeze-tolerant Cope's Gray Treefrog, Dryophytes chrysoscelis

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OSMOTIC ACTIVATION OF SPERM MOTILITY VIA WATER FLOW THROUGH AQUAPORINS IN THE FREEZE-TOLERANT COPE'S GRAY TREEFROG, *DRYOPHYTES CHRYSOSCELIS.*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By:

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Deja Miller ENTITLED Osmotic activation of sperm motility via water flow through aquaporins in the freeze-tolerant Cope's Gray Treefrog, *Dryophytes chrysoscelis* BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Miller, Deja. M.S. Department of Biological Sciences. Wright State University, 2018. Osmotic activation of sperm motility via water flow through aquaporins in the freeze-tolerant Cope's Gray Treefrog, *Dryophytes chrysoscelis.*

Gametes of gray treefrogs, *Dryophytes chrysoscelis*, are deposited into freshwater ponds. Sperm undergo spermatogenesis and maturation beginning in the seminiferous tubules and migrating to the lumen. In mammals and fishes, these cells are immotile within the isosmotic fluid of the testes and have motility activated by exposure to a hyper- or hypoosmotic medium. Water flows into or out of the sperm cell, altering intracellular ionic concentrations, and ultimately stimulates flagellar movement. We tested the hypothesis that exposure to a hypotonic environment activates motility of gray treefrog sperm. We also hypothesized that osmotic water uptake is facilitated by expression of water channel proteins from the aquaporin family. To test these hypotheses, we collected sperm from captive treefrogs maintained with food and water at 22°C and assessed motility of sperm immersed in hypoosmotic solutions of 200mOsm/L, 100mOsm/L, 75mOsm/L, 50mOsm/L, 25mOsm/L, and 10mOsm/L. A significant peak in reactivation percentages was seen at 50mOsmL (two-way ANOVA: $P<0.0001$, $F_{(6,24)}=20.64$). Motility was scored based off of projectile patterns, and were denoted as forward moving, wobblers, circular movers, and tail movers. Osmotic activation had a significant effect on forward movers (One-way ANOVA: $P<0.0001$, $F_{(6,28)}=9.093$), as well as circular movers (One-way ANOVA: $P=0.0307$, $F_{(6,28)}=2.766$). We looked for the significance of temperature on motility activation and viability; cooler temperatures (4°C) did not affect flagellar activation when compared to room temperature (22°C). However, cooler temperatures did prolong viability over a period of 24h (two-way repeated measures ANOVA: $P<0.0001$, $F_{(1,12)}=568.7$). We also assessed expression of mRNA

and protein for two aquaporins, HC-1, a homolog of the water channel AQP1, and HC-7, a homolog of the glyceroporin (glycerol/water channel) AQP7, in sperm and testes from those warm-acclimated animals and from animals that were cold-acclimated during the autumn and winter. We detected mRNA via PCR for both HC-1 and HC-7 in testes from warm-acclimated, cold-acclimated, and post-freezing thawed animals, but not in emitted sperm. At the protein level, immunolocalization of HC-1 in a cross section of warm testes indicated protein expression in the mesentery surrounding each testis, in the epididymis, and in interstitial cells. No HC-1 expression was evident in the immature spermatogonia or in mature spermatozoa within the tubular lumen. HC-7 in warm testes was expressed in the interstitial tissues of the testes and, at low levels, in primary spermatocytes within the seminiferous tubules of testes. No HC-7 expression was detected in mature sperm cells. Western blot analysis concurred with both RNA and IHC results showing a presence of both HC-1 and HC-7 in liver and various testes conditions, however no protein was detected in ejaculated sperm. We conclude that treefrog sperm require osmotic activation to acquire motility. That water uptake likely is achieved via water channel proteins, but the specific aquaporins that are involved remains to be confirmed.

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I. Introduction

Spermatogenesis gives rise to mature haploid spermatozoa via mitotic and meiotic divisions of primordial germ cells (Boj et al. 2015; Rugh 1951). Within the testes, spermatogonia begin their mitotic divisions in the seminiferous tubules formed by Sertoli cells (Boj et al. 2015). Spermatogonia mature into primary and secondary spermatocytes and eventually become spermatozoa; this maturation process begins in the basement membrane of the seminiferous tubules and progresses as cells into the lumen of the respective tubule (Rugh 1951). Maturation of sperm relies upon gonadotropins produced in the anterior pituitary and androgens produced by testicular interstitial Leydig cells (Rastogi et al. 2011). In north temperature anuran species, hormone release and sperm maturation is seasonal, associated with the animals' annual reproductive cycle. In these discontinuous reproductive cycles, species' spermatogenesis is completely interrupted during the colder months. Testes become non-responsive to either hormonal or environmental stimuli; the testes reduce in mass, and spermatogenesis cannot be stimulated by any means during this period of stasis (Rastogi et al. 2011). When spermatogenesis is a seasonal process, sperm maturation is completed in time for hibernation; winter testes contain few immature spermatogonia and mainly exhibit clusters of mature spermatozoa bundles (Rugh 1951).

In contrast, continuous anuran male reproductive cycles are not limited by seasonality; nevertheless, potentially continuous cycles may be limited by seasonal stimuli like rainfall, and testes may show variation in spermatogenic stages throughout the year. Even in species with potentially continuous reproduction, males may maintain a strict breeding season and show a moderate to strong reduction in spermatogenesis during certain months (Rastogi et al. 2011).

In Cope's gray treefrogs, *Dryophytes chrysoscelis* (formerly *Hyla chrysoscelis*) (Duellman, Marion & Hedges 2016) mating is activated by warming Spring temperatures and rainfall and may continue through spring and summer. Fertilization itself occurs externally, as male and female animals come together to release their gametes into shallow water. In aquatic vertebrates with external fertilization—including fishes and amphibians—the sperm that are released at the time of fertilization have not yet achieved full motility (Boj, Chauvigné, and Cerdà 2015). Rather, it appears that the osmotic environment within the animal suppresses motility, and full motility is acquired by osmotic activation from the external medium, whether in hyper- or in hypoosmotic waters.

Following spermatogenesis in marine fishes, sperm remain quiescent within the testes in ~300mOsm kg^{-1} isosmotic seminal fluid (Byrne 2015; Chen et al. 2011; Edwards 2004; Morisawa, Suzuki & Morisawa 1983; Morisawa et al. 1983); this may save the spermatozoon energy for swimming and fertilization (Christensen 2004). Motility activation in fishes that expel their sperm into seawater, such as the teleost *Sparus aurata,* is initiated by a strong hyperosmotic (Δ ~700-900 mOsm) shock immediately following deposit into seawater (Boj, Chauvigné & Cerdà 2015). This osmotic shock is thought to play an important role in the activation of sperm (Byrne 2015; Chen & Duan 2011; Chen et al. 2011; Christensen 2004); diffusion of water out of the cell caused by hyperosmotic shock signals activation of motility (Boj, Chauvigné & Cerdà 2015; Christensen 2004). The mechanistic link between osmotic shrinkage and activation of motility has not been clearly defined. However, increasing intracellular [Ca^{2+}] and [K^{+}], induced by osmotic water loss, and potentially a change in intracellular pH may activate flagellar movement (Boj, Chauvigné & Cerdà 2015).

In contrast, for amphibians and freshwater fishes, motility of healthy viable sperm, required for successful fertilization, is initiated by the sperm's response to a hypoosmotic environment (Edwards 2004; Byrne 2015, Chen & Duan 2011; Chen et al. 2011). In freshwater fishes the isosmotic 300mOsm kg^{-1} seminal fluid within the sperm ducts (Morisawa, Suzuki & Morisawa 1983; Morisawa et al. 1983) immobilizes motility, as similarly seen in marine fishes. Upon being deposited into a hypoosmotic environment, sperm of freshwater fishes and anurans experience an influx of water which alters intracellular calcium and potassium concentrations. Interestingly, sperm activation by hypoosmotic shock also occurs in mammals with internal fertilization. In mice, for example, sperm cells are immobilized in a 415 mOsm environment within the epididymis. Subsequently, they venture into a relatively hypoosmotic 310 mOsm environment within the uterine cavity (Chen et al. 2011) where motility is activated. Activation of sperm motility thus requires osmotic fluxes of water across the sperm membrane.

Cellular efflux or influx of water is achieved via aquaporins. Aquaporins are transmembrane proteins that facilitate the movement of water and other small solutes in and out of the cell (Zilli 2009). A six-transmembrane $\propto$-helical monomer folds and orients itself to form a pore (Dong 2016). Each monomer contains interlocking and highly conserved asparagine-proline-alanine domains (NPA) (Dong 2016; Zilli 2009). In the cell membrane, four of those monomers associate to form a tetramer with four respective pores (Sherman 2008; Zilli 2009) through which the flow of water and/or small solutes occurs. Aquaporins are constitutively open (Stogsdill 2017) and utilize diffusion and osmosis for flow. To date, thirteen mammalian aquaporins (-0 through -12) have been identified. These proteins form three sub-families: aquaporins (AQP), glyceroporins (GLP), and super aquaporins (Shivaraj 2017; Skowronski 2009). AQP proteins (-0, -1, -2, -4, -5, -6, -8) transport water, and in some cases, small solutes

such as ammonia or CO_2 (Skowronski 2009; Zilli 2009). GLPs (-3, -7, -9, -10) transport glycerol and potentially other small neutral solutes across the membrane along with water, though typically with lower water permeability coefficients than for AQPs (Zilli 2009; Zimmerman 2007). The final family, super aquaporins (-11, -12), contain poorly conserved asparagine-proline-alanine sequences (Skowronski 2009) and have no known function (Finn 2014).

Sperm and testicular cells of many animals express aquaporins. The marine teleost *Sparus aurata* expresses AQP-1aa in the flagellum, -7 in the head of ejaculated spermatozoa, and -8b in the mitochondria in motile sperm cells (Boj, Chauvigné & Cerdà 2015; Boj et al. 2015; Zilli 2009). Demonstrated by immunohistochemical assays, AQP-1ab, -8b, and -10b are localized in both the head and anterior tail (Boj, Chauvigné & Cerdà 2015; Boj et al. 2015; Zilli 2009). Expression of AQP-3 was detected in mouse sperm and testes at both the mRNA and protein levels. Immunohistochemistry shows localization of AQP-3 in the cauda epididymis in the testes, and also in tails of ejaculated sperm (Chen et al. 2011). AQP-3 also localizes to sperm tails within the epididymis during late spermatogenesis (Chen et al. 2011). As previously mentioned, these proteins are thought to play a role in osmotic regulation that initiates motility (Byrne 2015, Chen et al. 2011, Christensen 2004). In addition to motility activation, aquaporins may contribute to spermatogenesis (Skowronski 2009). In White Koluda Geese expression of AQP-1 and -5 in the capillary endothelium and -7 in the tails of spermatozoa have been verified with immunolocalization in the testes and vas deferens (Skowronski 2009). In rats AQP-0, -1, -3, -7, -8, and -9 have been identified in the testes (Chen et al. 2011, Skowronski 2009) and AQP-1, -9, and -10 have been identified in the efferent ducts (Skowronski 2009). This supports the concept that aquaporins are involved in early stages of spermatogenesis and in the secretion of tubule fluid (Skowronski 2009).

Aquaporin expression in sperm cells is thought to play an important role in sperm development, maturation, and motility activation. Water flow through these proteins alters intracellular ion concentration which stimulates the machinery needed for projection. However, optimal conditions outside of the cell maintain sperm motility and viability. A shift in intracellular pH has been thought to induce flagellar movement (Boj, Chauvigné & Cerdà 2015); environmental pH may have some effects on sperm motility as well. A study conducted in the African clawed frog, *Xenopus laevis*, showed that sperm motility increased within the range of 5.5-7.8 with a peak at 7.0 (Christensen *et al.* 2004). Human semen has a pH ranging from 5.2 to 8.2; sperm motility in acidic environments pH 5.2 and 6.2 was significantly reduced when compared with sperm incubated in pH 7.2 and 8.2 (Zhou *et al.* 2015). The range of pH shows an effect on motility induction but viability and velocity of the sperm is effected by temperature. In the Landrace-strain boar sperm flagellar speed preserved at 17°C was significantly slower than sperm incubated at 38.5°C (Kim *et al.* 2017). The Peron's tree frog, *Litoria peronii,* 75% of sperm stored at 23°C maintained viability after 120 minutes; at 4°C maximum longevity ranged from 80 hours to 320 hours (Sherman *et al.* 2008).

While aquaporin expression has been reported in the testes and sperm of a number of fishes, birds, and mammals, little is known of osmotic activation or aquaporin expression in amphibians. Furthermore, the effects of intracellular and extracellular factors on flagellar activation and sperm viability has not been extensively studied. *Dryophytes chrysoscelis*, commonly known as Cope's Gray Tree Frog, is an amphibian native to eastern United States with the unusual ability to freeze ~70% of its body water during winter and resume functioning post-thawing. During mating season (May-July), gray treefrogs congregate near small bodies of water, where they enter amplexus (mating pairs) in trees near or along the shallow ends of the

water (Sherman 2008). The frogs then deposit eggs and sperm into the freshwater for external fertilization (Christensen 2004). Unlike the oocytes deposited in freshwater (Edwards 2004), sperm cells do not contain a protective jelly coating to prevent cellular swelling from the hypoosmotic environment (Christensen 2004). Instead, as noted above, osmotic influx of water is likely required to activate motility, and that water influx is likely via aquaporins. I hypothesized that a) sperm from *D. chrysoscelis* would show an increase in motility when exposed to hypoosmotic shock, with the peak percentage of motility occurring at the lowest osmolarity, and that b) sperm motility and viability are temperature dependent, with a higher percentage of reactivation and prolonged viability in colder temperatures. Furthermore, I hypothesized that osmotic activation of motility could be accounted for by expression of AQP-1 in sperm tail and AQP-7 in the sperm head. I hypothesize that *D. chrysoscelis* exhibits a potentially continuous spermatogenic cycle and therefore has various stages of sperm within its testes year-round; aquaglyceroporin (AQP-7) expression in these tissues during cold-acclimation, freezing, and thawed conditions should rise in response to decreasing temperatures to protect these tissues from cryoinjury.

II. Materials and Methods

Animal collection and housing

Male gray treefrogs were captured from ponds in Southwest Ohio in May and June during their breeding season and were identified based on their trill frequencies (Stogsdill 2017, Zimmerman 2007). Captive animals were housed in Wright State University's Laboratory Animal Resources facility in plastic cages (12"x6"x6") with free access to water and crickets 2-3x weekly. During the summer months, all frogs were maintained at 20°C with a 12:12 (light:dark) light cycle. Around the start of fall, 16 frogs were moved to a walk-in cooler for cold acclimation. Warm frogs remained in the 20°C room. Frogs who were cold acclimated were transferred to a temperature-controlled room that was progressively cooled to 5°C with a 8:16 (light:dark) light cycle over a period of two months. Cold-acclimated frogs remained in this condition for 4-6 weeks before being sacrificed for tissue collection. Frozen frogs were first cold acclimated and then exposed to a further decrease in temperature from 5°C to -2.5°C over a period of one week. Freezing was initiated by touching a piece of ice to the animal's surface. Frogs were left frozen for 24 hours before being sacrificed. Thawed frogs were taken through the cold and frozen protocols and then returned to 5°C for 24 hours before tissue collection (Stogsdill 2017).

Sperm and testes collection

Sperm ejection was induced by injection of gonadotropin hormone (Hopkins & Herr 2017; Waggener & Carroll Jr. 1998). Lyophilized human chorionic gonadotropin was suspended in MilliQ water and then diluted in a Ringer's solution (Ringer's; in g/L: NaCl 8.1, KCl 0.15, $CaCl_2$ 0.15, $NaHCO_3$ 0.2) to a final concentration of 200IU in 200µl (Hopkins & Herr 2017).

The 200µl aliquot was drawn into a 1cc syringe with a 27-gauge needle. Frogs were held on their dorsal side on a dissecting tray covered in a wet paper towel and their abdomens were wiped with an alcohol prep pad. The hCG was injected intraperitoneally. Frogs were then placed in a plastic container containing a shallow layer of water for two minutes, then moved to a second plastic container containing a wet paper towel lining the walls to keep a humid environment. Following a one hour and fifteen-minute incubation period, the frogs' cages were checked for sperm-containing urine. If urine was present in the housing, it was pipetted and saved in a room temperature micro-centrifuge tube. Frogs that did not expel their urine freely had a capillary tube inserted into their cloaca to collect the sample. Collections occurred each hour following the initial one hour and fifteen-minute wait for a total of three collections. Each individual frog recovered for at least two weeks between injections. Frogs were caught and placed in captivity in May or June and injections and subsequent collections occurred between July and April of the following year. Warm acclimated frogs continued to respond to hCG injections with sperm release throughout those months.

Testes from warm, cold, and thawed conditions were collected for mRNA (n=3 per condition) and protein (n=3) extraction, as well as for immunohistochemistry (n=3). Frogs were killed and tissues were immediately flash frozen in liquid nitrogen or on dry ice, and stored at -80°C. A lobe of liver (n=3) was also flash frozen to be used as a control sample. Warm-acclimated, cold-acclimated, and thawed testes and lobes of liver also were placed in buffered formalin and, after processing, embedded in paraffin for IHC analysis (Stogsdill 2017). Testes from frozen animals were not used due to insufficient numbers.

Motility Analysis of Sperm

Immediately following sample collection, 2µl of sperm cells were observed for motility analyses. During collections, some motility activation could have occurred due to sperm exposure to hypoosmotic urine. Samples in which greater than 50% of sperm showed motility upon collection were further tested; samples that showed less than 50% total motility or no motility were discarded. This is due to low or non-motile samples incapability of being reactivated. The cause of the inability to reactivate is not known; a potential reasoning is inhibitory ion concentrations. As seen in the European burbot, *Lota lota,* high intracellular concentrations of K^+ ions or NaCl can eliminate motility (Dziewulska and Pilarska 2018). Samples were then placed on ice to prolong viability. Motility assays and the response to osmotic activation were then initiated within 5 minutes. To do so, sperm were first brought to a uniform inactive state by placing 10µl of motile sperm into 40µl "non-activating medium" (NAM; 290 mOsm Ringer's solution, which has been shown previously to inactivate motility; Byrne 2015) for 5 minutes. To potentially reactivate sperm motility, 2µl sample of the inactivated sperm cells in NAM were pipetted onto a pre-cleaned slide and viewed to ensure cessation of motility. 18µl of diluted Ringer's solution (200mOsm, 100mOsm, 75mOsm, 50mOsm, 25mOsm, 10mOsm) then were pipetted directly onto the 2µl of inactive sperm and allowed one minute to reactivate. Following the one minute reactivation period, 20 second videos were recorded and subsequently assessed.

To quantify motility, I determined the overall percentage of motile cells and I characterized their patterns of motility. Motility patterns were categorized as forward movers, wobblers, tail movers, and circular movers (Boj, Chauvigné, and Cerdà 2015). Sperm that projected forward in a linear fashion were deemed forward movers. Wobblers were classified as

sperm that moved from side to side or appeared to vibrate but did not move forward or change position. Tail movers were sperm whose flagellum whipped from side to side or in a circular fashion but the head did not show signs of movement, nor did the sperm change position. Circular movers were sperm whose head and tail moved together but appeared to rotate rather than move a distance.

To assess the effect of temperature on reactivation, freshly collected sperm were incubated at room temperature or at 4°C for one hour before being tested. To analyze viability over time, cells were incubated at room temperature or at 4°C absent of NAM. At time points of 0, 2, 4, 8, 12, and 24 hours, 2µl of cells from each condition were viewed to score the number of cells still showing motility. Only samples in which at least 50% of motility was activated upon collection was used.

Analysis of aquaporin mRNA expression

Total mRNA was extracted from sperm cells via the Qiagen RNeasy mini-kit. Fresh sperm samples were centrifuged at 300g to form a pellet and the supernatant was removed. To disrupt the cells, 600µl of Buffer RLT was added to the pellet, mixed via vortex and passed through a 20-gauge needle five times. A 600µl volume of 70% ethanol was added to the tube and the entire sample was transferred to an RNeasy spin column. The sample was centrifuged at 8,000g for 15s and 700µl of Buffer RW1 was added. The column was again centrifuged and 500µl of Buffer RPE was added and spun twice. The column was placed in a new RNase free tube and the RNA was eluted into the tube in 30µl of RNase free water.

Total mRNA was extracted from the tissues using Trizol reagent. The tissues were disrupted in a glass homogenizer containing 1ml of Tri-Reagent while on ice. Homogenate was

transferred to a fresh micro-centrifuge tube and left at room temperature for 5 minutes. Chloroform (200µl chloroform to 1ml of Tri-reagent) was added to the mixture, shaken vigorously for 15s and left at room temperature for 3 minutes. The sample was then centrifuged at for 15 minutes at 12,000g at 4°C. The upper aqueous phase was placed into a new tube with 500µl of 100% isopropanol and incubated at room temperature for 10 minutes. The sample was spun at 12,000g at 4°C for 10 minutes. The supernatant was discarded and 75% ethanol in DEPC water was used to wash that pellet for 5 minutes at 7,500g. The pellet was air dried for 5 minutes and then suspended in 30µl DEPC water.

Synthesis of cDNA from either sperm or tissue mRNA samples was done via the Invitrogen Superscript III First-Strand Synthesis System kit. RNA (5µg or 8µl) 1µl of Oligo dT primer, and 1µl of 10mM dNTP mix was incubated at 65°C for 5 minutes, then placed on ice for 1 minute. A cDNA reaction mix containing 2µl 10X Reverse Transcriptase Buffer, 4µl 25mM $MgCl_2$, 2µl 0.1M DTT, 1µl RNaseOUT, and 1µl Superscript III Reverse Transcriptase, was added to the RNA mixture and incubated at 50°C for 50 minutes. Samples were also incubated without reverse transcriptase for a negative control. RNase H was added to each tube and incubated at 37°C for 20 minutes to destroy the original mRNA template.

Polymerase Chain Reaction was carried out using the New England BioLabs Taq DNA polymerase with standard Taq buffer kit for a 25µl sample. Each reaction contained 2.5µl Taq buffer, 0.5µl 10mM dNTP, 0.125µl Taq polymerase, 19.875µl water, 1µl cDNA sample, and 0.5µl of both 10mM forward and reverse primers. Primers were specific for *D. chrysoscelis* AQP-1, and -7. For AQP-1, the forward and reverse primers were 5'-TACACTGGGTGCGGTATGAA-3' and 5'-TCGTACTCCTCCACCTGTCC-3' respectively. For AQP-7, the forward and reverse primers were 5'-ATTGTCGGCCTCCTGGTTAC-3' and 5'-

ACCCAGCACCAGTAATTCCC-3' respectively. Each primer set amplified a fragment of ~200 base pairs. Primers for β-actin were used to ensure quality cDNA was collected. For β-actin, the forward and reverse primers were 5'-CAGATCATGTTTGAGACCTC-3' and 5'-GTCACACCATCACCAGAGTC-3' respectively. PCR was carried out in an Eppendorf Mastercycler Gradient Thermocycler with an initial denaturing step at 94°C for 3 minutes, followed by 40 cycles of 94°C for 45 secs, 54°C for 45 secs, 72°C for 1 min, and ending with a final elongation step at 72°C for 5min (Stogsdill 2017). A 10µl sample of the PCR product was run on a 2% agarose gel containing 2µl of ethidium bromide. Images were taken with the Amersham Imager 600 UV.

Analysis of aquaporin protein expression

Total protein was extracted from sperm and tissues by adding 356µl lysis buffer (50mM Tris-HCl, 100mM NaCl, 1mM EDTA pH 8.0, 10% glycerol, pH to 7.4) and 4µl Protease inhibitor cocktail (Sigma, P8340) and homogenized with an electronic homogenizer. To each sample, 40µl of 10% Triton-X was added and mixed every 10 minutes for 30 minutes on ice. Centrifuging pellets at 10,000g for 5 minutes at 4°C removed insoluble material in the solution so that the protein-containing supernatant could be stored separately. Total protein concentration was determined via BCA assay.

Protein expression was analyzed via Western blotting using SDS-PAGE gels composed of a 4% stacking gel and 12% resolving gel. Each well was loaded with 30µg of protein. The mini tank was filled with electrophoresis buffer (in g/L: Tris base 3.03, Glycine 14.4, SDS 1.0g) and run at 100V. The transfer cassette was then assembled using PVDF membrane run at 200mA for 90 minutes in ice-cold transfer buffer (in g/L: Tris Base 3.03, Glycine 14.4, Methanol

200ml). PVDF membrane was then blocked in 5% milk in TBST (100mM Tris-Cl pH 7.5, 150mM NaCl, 0.05% Tween-20) for one hour. Primary antibody solutions were made with rabbit anti-HC-1 and rabbit anti-HC-7 polyclonal antibodies in 1% milk in TBST at 1:1000 dillutions. Peptide competition assays were performed to show specificity of the antibody to the antigenic sequence by incubating the antibody solution with a 200-fold molar excess of the immunizing peptide for one hour before application to the membrane. Primary antibodies and respective peptides were poured over the membrane in parafilm wrapped dishes containing the blots, and left to rock overnight at 4°C. TBST was used to wash the blot, followed by an incubation in the secondary antibody (1:5000) in 1% milk for one hour. For HC-1 and HC-1, donkey anti-rabbit secondary antibodies were conjugated with horseradish peroxidase for rabbit antibodies. Donkey anti-mouse secondary antibodies were conjugated with horseradish peroxidase for mouse in β-actin. The blot was imaged using chemiluminescence detection using the ThermoScientific Pierce ECL Western Blotting Substrate kit. Images were taken with the Amersham Imager 600 UV.

Immunohistochemistry

To identify and localize aquaporin expression, sperm cells were spread onto Fisherbrand Superfrost Plus microscope slides and fixed with 95% methanol. Cells were permeablized in Triton-X 100 and blocked in 10% BSA blocking serum (in 10ml; BSA 0.4g, Donkey serum 1ml, Tween-20 5µl, brought to volume with PBS pH 7.2). Slides were incubated with primary antibody (1:100) in 1% blocking serum overnight at 4°C. Intermediate washes were done with 0.1M PBS (0.02M sodium mono-basic phosphate, 0.08M sodium dibasic phosphate, 150mM NaCl, pH 7.2). Secondary antibody (1:500), conjugated to either FITC or Cy5 as a fluorescence

tag, was applied in 1% blocking serum and incubated for 1.5 hours. Mounting medium containing DAPI staining was also applied to visualize the cell nucleus. Slides containing the Cy5 marker were imaged via the Olympus FV1000 confocal microscope; slides containing the FITC were imaged via a Nikon TE2000-S inverted fluorescence microscope with a Photometrics CoolSNAP ES camera and Metamorph software (version 6.1r4).

For immunohistochemistry of testes, tissues were embedded in paraffin molds and 10μm sections were mounted on Fisherbrand Superfrost Plus microscope slides. Deparaffinizing and hydration occurred through a series of two ten-minute xylene washes, two five-minute 100% ethanol washes, one five-minute 95% ethanol wash, and one five-minute 80% ethanol wash, followed by heat-induced epitope retrieval from aldehydes (30 minutes in 95° citrate buffer: Citric acid anhydrous 1.92g, Tween-20 0.5ml, pH to 6.0) (Boj 2015). Sections were washed in 0.1M PBS and incubated in 0.2% Triton X-100, 1% glycine, and 0.1% $NaBH_4$ for 15-minutes each. Blocking in 10% BSA blocking solution followed by overnight incubation of primary antibody (1:100) in 1% blocking serum occurred as in sperm slides. The secondary antibody contained the Cy5 flurophore, and fluorescence microscopy proceeded as described above.

Statistics

Statistical analysis was done with Graphpad Prism 7 version 7.0d. Data were imported into the grouped tables. A two-way ANOVA was used to test significance for the effects of individuals and of medium osmolarity on sperm motility. A two-way repeated measures ANOVA was used for testing the effects of temperature on osmotic activation, as well as temperature effect on viability overtime. Post-hoc tests were performed on significant results. For all statistical testing, significance was assumed if means differed with $P<0.05$.

III. Results

Effect of activation-medium osmolarity on sperm activation

Motile sperm were successfully inactivated in NAM with an osmolarity of 290 mOsm/L. Reactivation of motility began after dilution with solutions having osmolarities as high as 200 mOsm/L. As predicted, the percentage of cells that regained motility increased as the medium became more hypoosmotic (fig. 1A). Unexpectedly, though, the percentage of motile sperm reached a peak with a dilution osmolarity of 50 mOsm/L and then decreased when the diluent osmolarity was 25mOsm/L and 10mOsm/L (One-way ANOVA: $P<0.0001$, $F_{(6,28)}=22.10$). A Dunnett's multiple comparisons test showed that osmolarities of 100mOsm/L to 10mOsm/L varied from 290mOsm/L.

"Forward movers" was distributed similarly to total motility (fig 1B); peak occurrence of this motility pattern occurred at 50 mOsm/L (One-way ANOVA: $P<0.0001$, $F_{(6,28)}=9.093$). However, a Bonferroni's multiple comparisons test showed that only osmolarities of 100mOsm/L to 50mOsm/L were significantly different than the inactive 290mOsm/L. Circular movers (fig. 1D) were also significantly affected by osmolarity (One-way ANOVA: $P=0.0307$, $F_{(6,28)}=2.766$). A Dunnett's multiple comparisons test showed 75mOsm/L and 50mOsm/L were significantly different than 290mOsm/L. Wobblers (fig 1C) showed no significant difference in reactivation between the osmolarities (One-way ANOVA: $P=0.1520$, $F_{(6,28)}=1.725$). Similarly, tail movers (fig. 1E) (One-way ANOVA: $P=0.7058$, $F_{(6,28)}=0.6289$) did not vary significantly in occurrence across osmolarities.

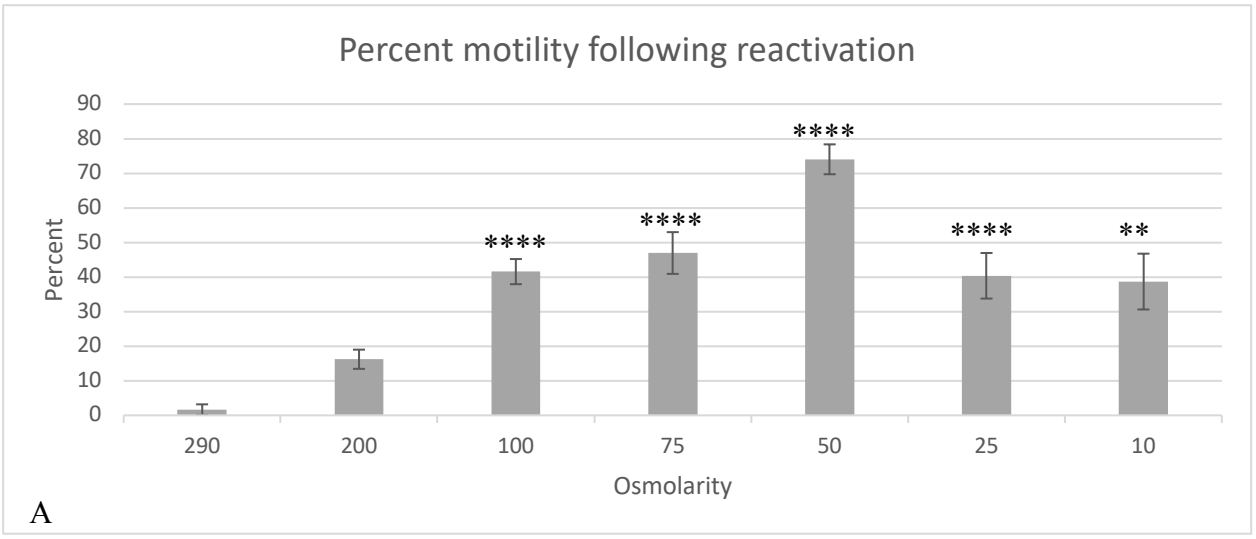

Figure 1A. Effect of diluent osmolarity on motility of sperm previously inactivated in NAM. Peak reactivation occurs at 50mOsm/L ($P<0.0001$, two-way ANOVA). ** has an adjusted $P=0.0019$, **** $P<0.0001$. N=5. Bars are +/- SEM.

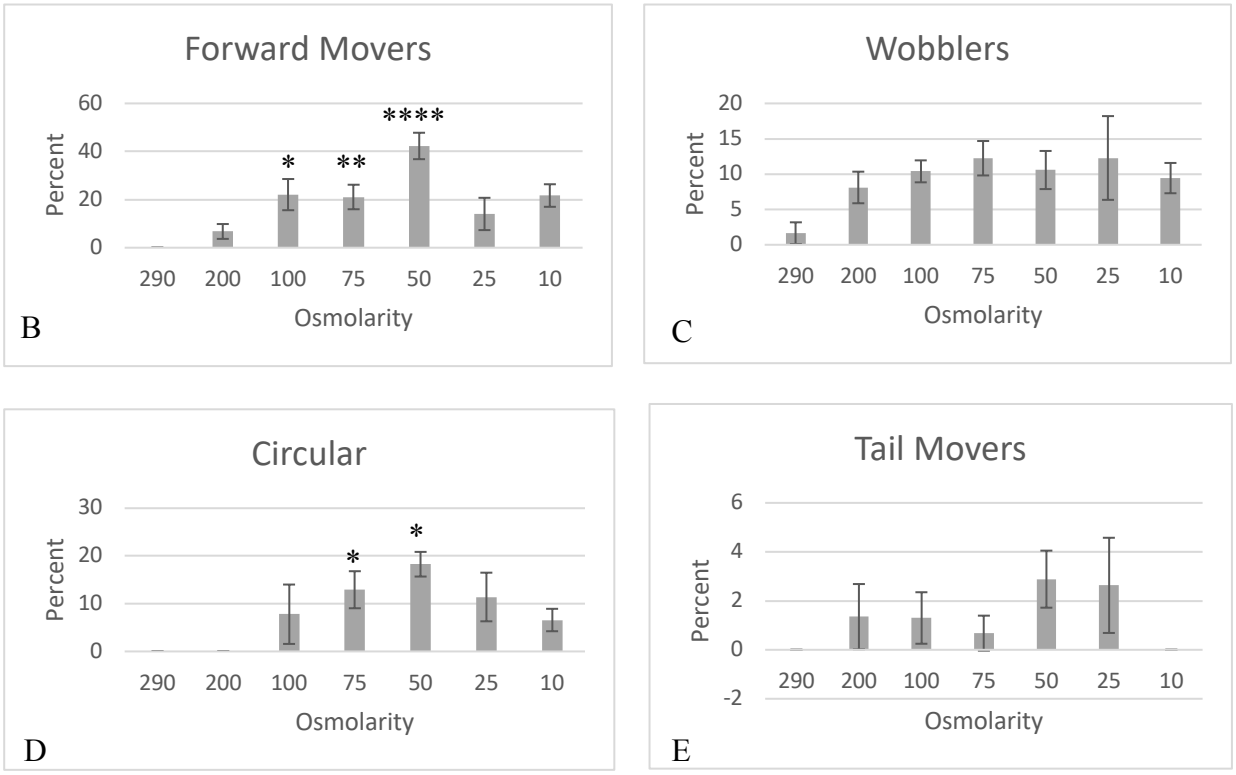

Figure 1B-E. Percent of reactivated sperm that exhibited each of four motility types. Diluent osmolarity had an effect only on forward movers. (B): * $P=0.0363$, ** $P=0.0088$, **** $P<0.0001$. (D): *=0.0449, *=0.0486. N=5 samples. Note the differences in Y-axis ranges. Bars are +/- SEM.

Effect of temperature on osmotic activation of sperm motility

Sperm incubated at 4°C for an hour showed reactivation only at osmolarities of 50 mOsm/L to 10 mOsm/L, which varies from the previous results where activation of fresh samples occurred as high as 200 mOsm/L. Sperm at room temperature showed reactivation at 100 mOsm (fig 2) which is more consistent with the previous results. A two-way ANOVA shows that there was no significant difference between reactivation between the two temperatures (Two-way repeated measures ANOVA: $P=0.1834$, $F_{(1,2)}=4.003$). A significant difference was seen between the osmolarities (Two-way repeated measures ANOVA: $P=0.0137$, $F_{(5,10)}=5.133$) which is consistent with the previous results.

Effect of temperature on viability over time

Sperm samples at both room temperature and 4° showed motile cells following a 24-hour incubation period (fig 3). However, sperm at 4°C maintained a significantly greater percentage of motility compared with sperm at room temperature (two-way repeated measures ANOVA: $P<0.0001$, $F_{(1,12)}=568.7$). A Sidak's multiple comparisons test shows that a significant difference in viability can be seen at 4hours and the remaining time values following that. Incubation time also had a significant effect on the percentage of viable sperm cells (Two-way repeated measures ANOVA: $P<0.0001$, $F_{(5,12)}=352.1$). Both cold and warm samples showed a significant difference in viability over time. At 4 hours, sperm maintained at room temperature began to show a significant decrease in total percentage; sperm maintained in the cold showed a decline in total percentage at 8 hours (Sidak's multiple comparisons test).

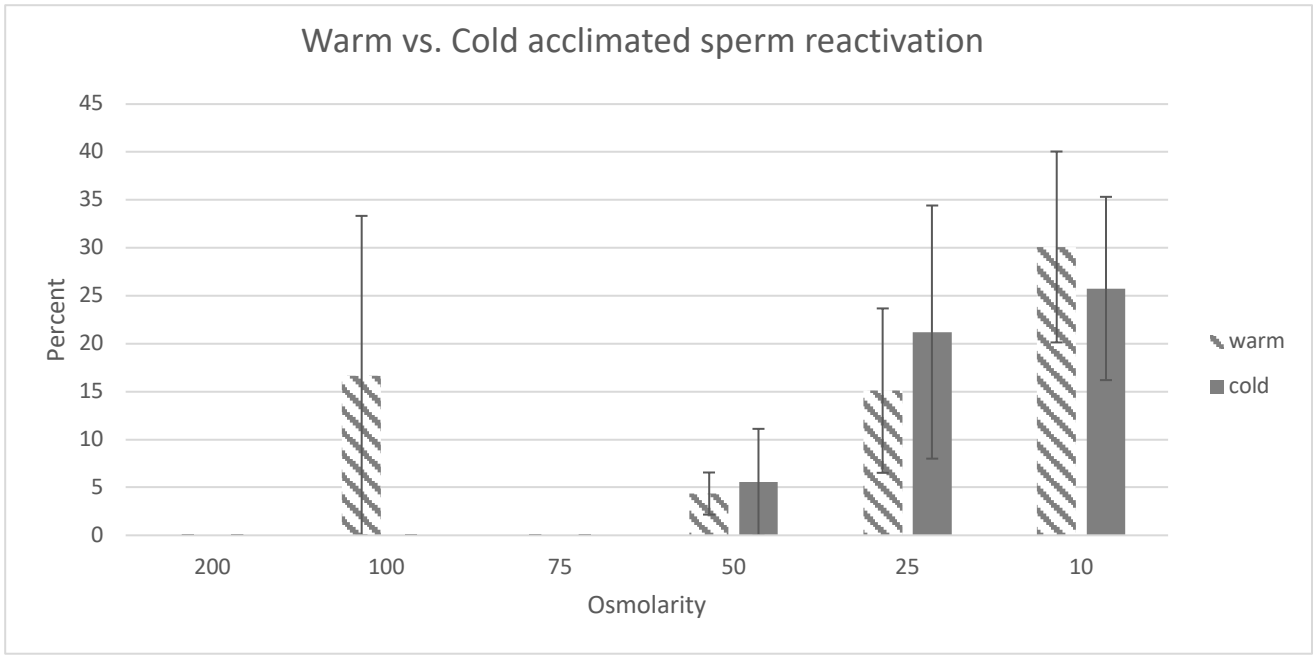

Figure 2. Effects of temperature on osmotic activation. Temperature had no effect on reactivation percentages. (P=0.1834, repeated measures ANOVA). N=3. Bars are +/- SEM.

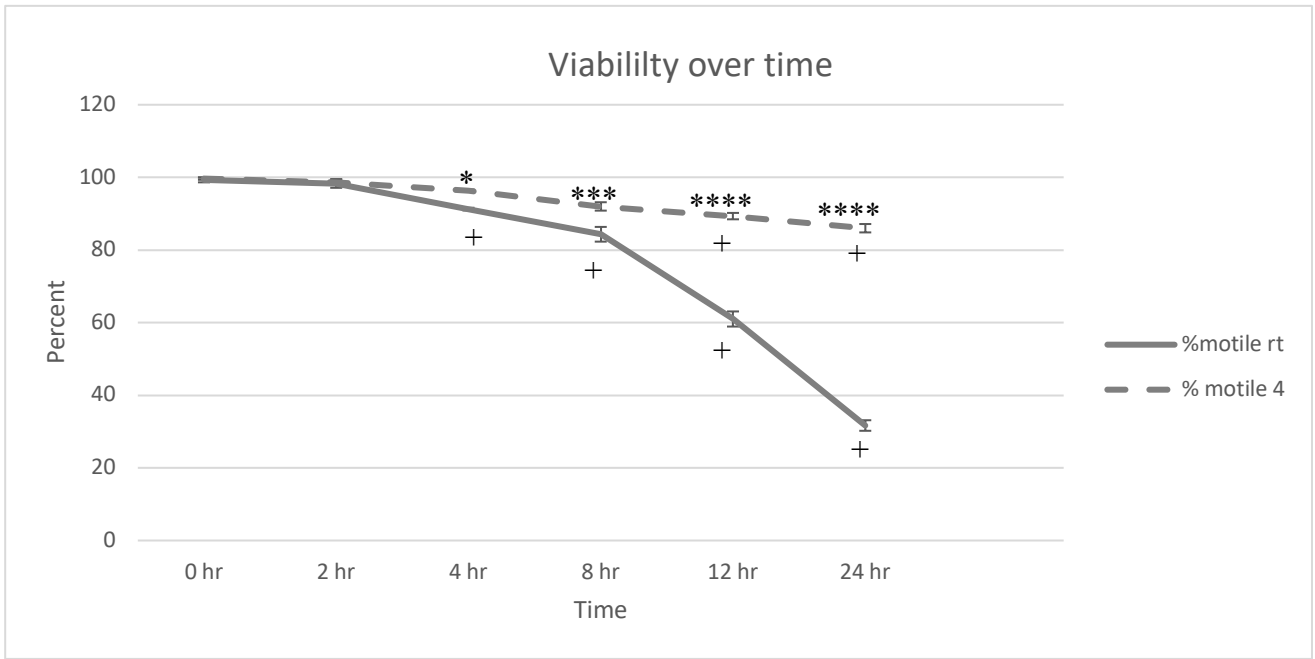

Figure 3. Effects of temperature on viability over 24 hours. Cells at 4°C maintained greater viability (P<0.0001, repeated measures ANOVA). N=3. Bars are +/- SEM. (*) Indicates significant differences between the temperatures. (+) Indicates differences between time values.

Aquaporin mRNA expression in Cope's gray treefrog sperm and tissues

PCR products of sperm, testes and liver mRNA were run on a 2% agarose gel and showed presence of HC-1 in testes in warm, cold, and thawed conditions as well as in the liver (positive control). Bands appeared at the 200bp size as expected. Expression of HC-1 did not appear in the sperm samples or in the sperm, testes, and liver samples lacking the reverse transcriptase serving as a negative control. Identical results for HC-7 were seen; bands were present in the liver and all conditions of the testes, but not in the sperm and –RT samples. HC-7 primer-dimers were also seen as represented by bands below 100 bps in every sample. Three replicates of each tissue were run and similar results were seen in each replication. PCR using β-actin primers also was completed in liver, sperm, and warm testes to ensure quality of RNA extraction. A band at ~150bps was seen in all three tissues, and was absent in the samples lacking reverse transcriptase. Primer-dimers were also seen in β-actin samples.

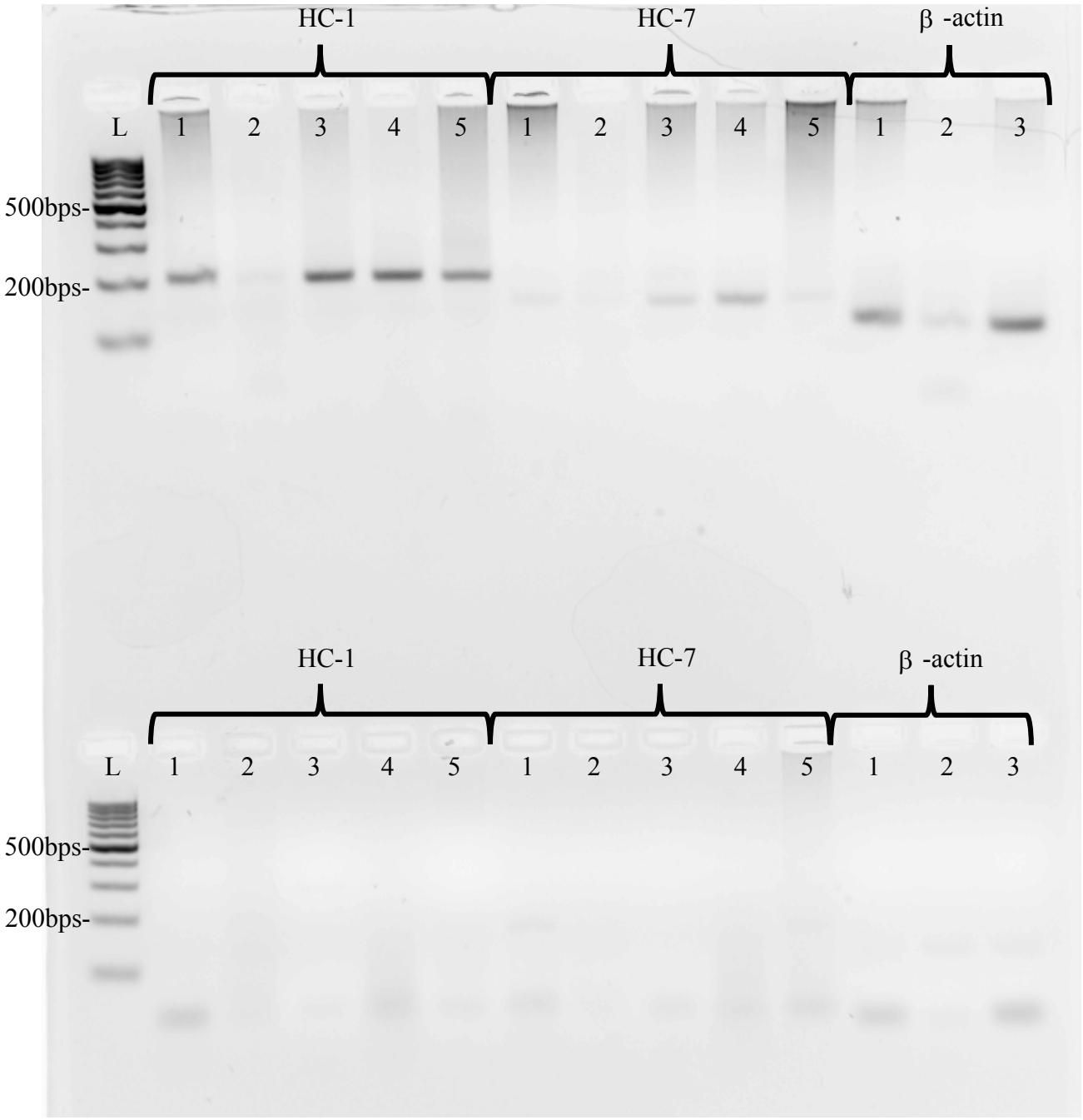

Figure 4. PCR amplification of HC-1, HC-7, and β-actin. Lanes represent (L) ladder, (1) warm liver, (2) sperm, (3) warm testes, (4) cold testes, (5) thawed testes. Top half of gel represents samples with reverse transcriptase; bottom half of gel lack reverse transcriptase.

HC-1 and -7 protein expression in reproductive tissues of Cope's gray treefrog

Western blot analysis of HC-1 expression showed protein expression in warm, cold, and thawed conditions of the testes (fig 5A). Liver was used as a positive control and showed presence of the protein. No banding was seen in Western blots of protein extracted from sperm. All bands were antibody-specific, as indicated by the absence of signal after pre-incubation of the antibody with the antigenic peptide. Peptide blocking successfully eliminated most bands while greatly reducing others. HC-1 protein has a nominal molecular mass of 28 kDa; as expected, Western blots of HC-1 yielded bands in all tissues right above the 26 kDa marker on the ladder. Western blots of extracts from cold and thawed testes and liver also produced a second band at ~55 kDa, suggesting a modified, perhaps glycosylated, form of the protein.

Aquaporin -7 analysis showed protein expression in cold and thawed testes and liver, but did not show signaling in the sperm (fig. 5B). Peptide blocking reduced but did not completely eliminate the signal. Aquaporin -7 protein is expected to measure at 31.7 kDa and all bands in Western blots of proteins extracted from testes appeared between the 26 kDa and 34 kDa markers on the ladder. Banding in liver appeared between ladder markers 34 kDa and 43 kDa.

Probing blots for β-actin as a check for successful protein extraction showed signal in the liver and all testes samples, but no banding was seen in the sperm sample. A dot blot of 10μg of liver and sperm protein was stained with Ponceau S to check protein collection. No protein was detectable in sperm, but protein was detected in liver (data not shown). Three replicates of each tissue were run and similar results were seen in each trial.

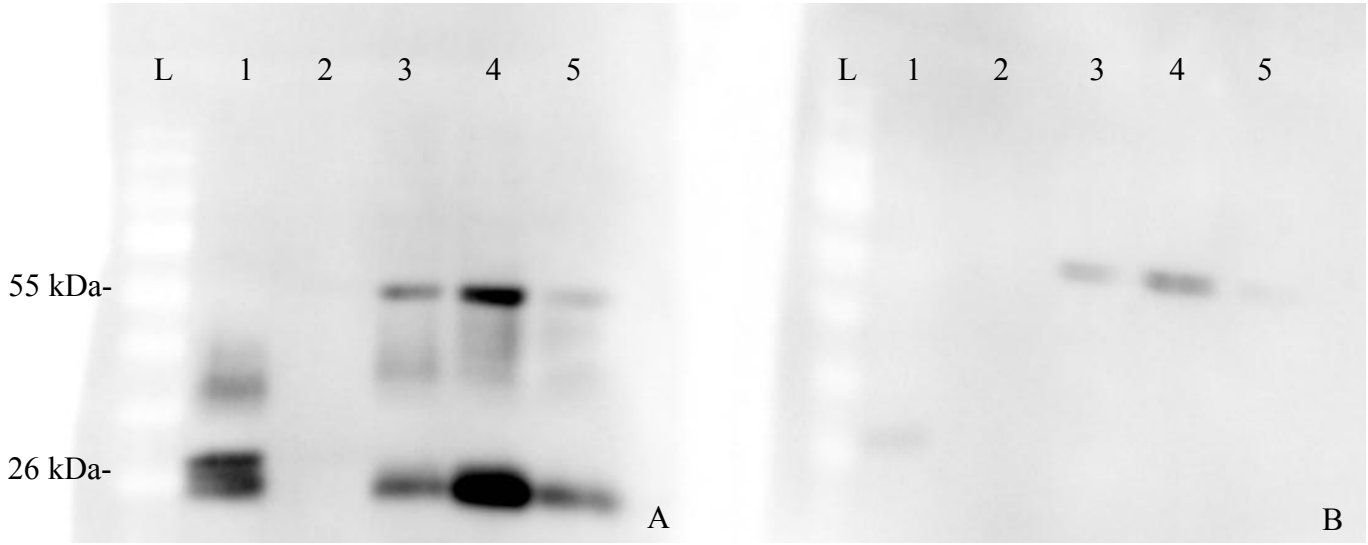

Figure 5A-B. Western blot analysis of HC-1. Lanes (in 5a and 5b) represent ladder (L), warm liver (1), sperm (2), warm testes (3), cold testes (4), thawed testes (5). Fig. 5a shows HC-1 expression in liver and all testes samples at the 26kDa marker; no signal is seen in sperm. Testes samples show an additional band around the 55kDa marker; liver shows an additional band around the 34 kDa marker. Fig. 5b shows a reduced expression of HC-1 from prior incubation with the antigenic peptide.

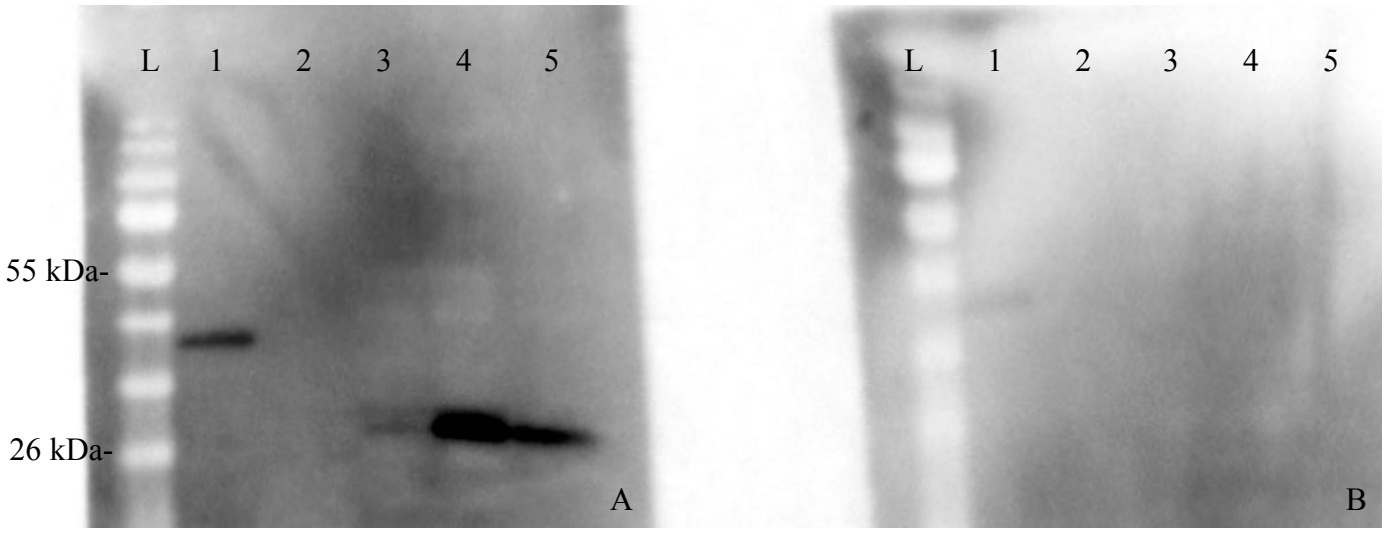

Figure 6A-B. Western blot analysis of HC-7. Lane labels are as in Figure 5. Fig 6a protein immunoblot detection in warm, cold, and thawed testes at MW above 26 kDa; for liver, the signal was between 34 kDa and 43 kDa. No signal was detected in sperm. Fig. 6b shows an eliminated signal of HC-7 from prior incubation with antigenic peptide.

Localization of aquaporin expression from IHC analysis

Immunolocalization of HC-1 in a cross section of warm testes shows protein expression in the mesorchium, a mesentery surrounding each testis, and the epididymis (Rugh 1951). Expression can also be seen in the interstitial cells. No expression was evident in the immature spermatogonia or in the mature spermatozoa within the lumen of the testes (fig. 7A-B). HC-1 in ejaculated sperm did not show expression in either the sperm head or the tail (fig. 7C). Sperm in various stages can be seen within each seminiferous tubule.

Immunolocalization of HC-7 in warm testes shows expression in the interstitial tissues of the testes and the seminiferous tubules (fig. 7D-E). No HC-7 detection could be seen in mature sperm cells within the tubules. HC-7 antibody did not show evidence of immunoreactivity in either the head or tail of ejaculated sperm (fig. 7F). A low level of immunoreactivity was detected in primary spermatocytes within the seminiferous tubules of testes (fig. 7D-E). HC-7 localized to the interstitial tissues in cold-acclimated, frozen, and thawed testes which coincides with the results seen in warm samples (fig. 8A-C). Hematoxylin and eosin staining of testes in warm, cold, frozen, and thawed conditions shows various stages of immature and mature sperm within the structures of the testes (fig 9A-H). Three replicates for each protein showed similar results.

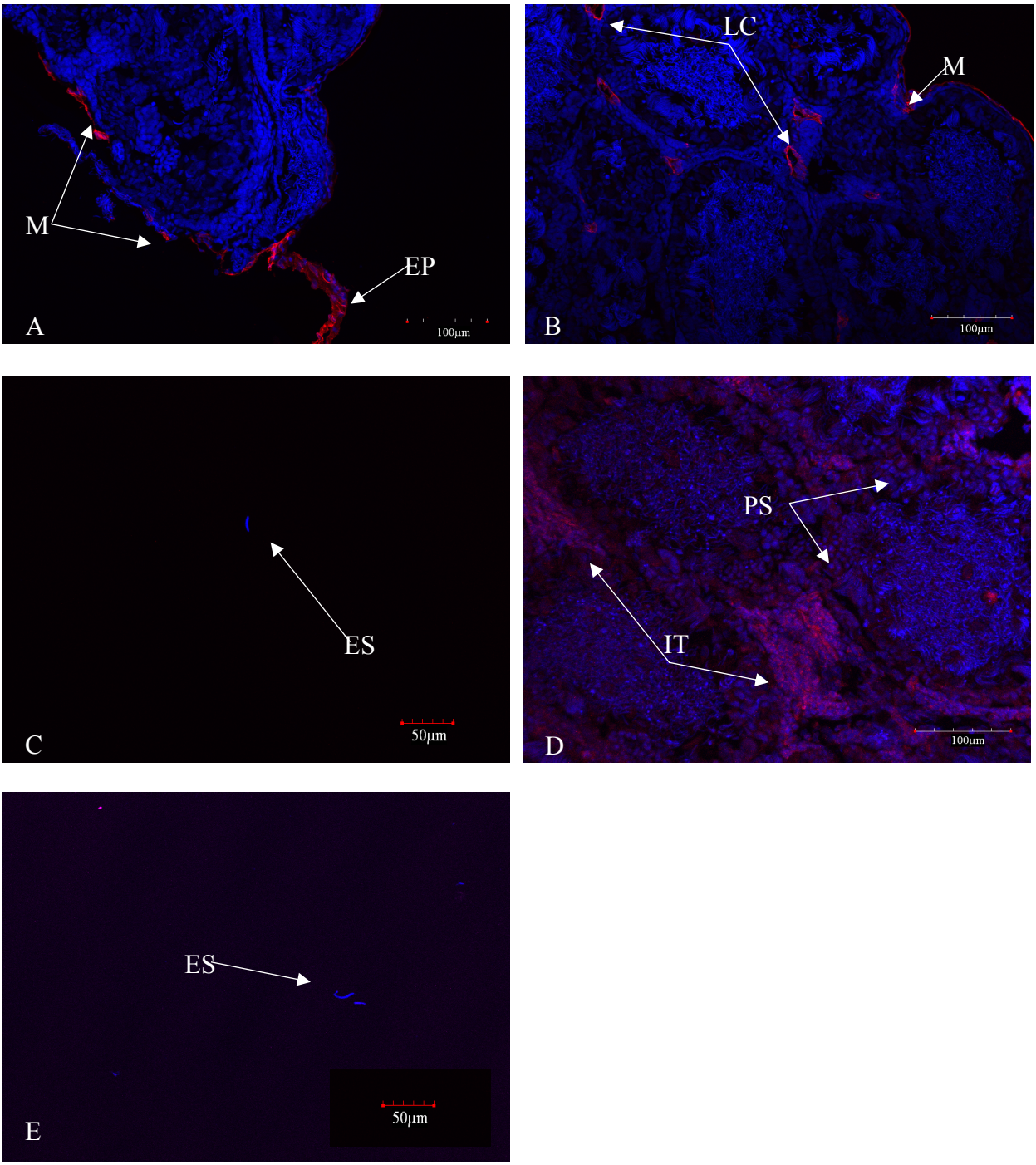

Figure 7. Immunohistochemistry analysis for protein localization. Panels (A) and (B) are cross sections of warm testes at 20X magnification for localization of HC-1 expression. Localization is in the mesorchium (M), epididymis (EP), and interstitial Leydig cells (LC). Panel (C) HC-1 is undetectable in ejaculated sperm (ES). Panels (D) is a cross section of warm testes at 20X magnification for localization of HC-7 expression. Signaling is localized to interstitial tissue (IT) and primary spermatocytes (PS). Panel (E). HC-7 is undetectable in ejaculated sperm (ES). Blue staining represents DAPI; red staining is the Cy5 or FITC flurophore.

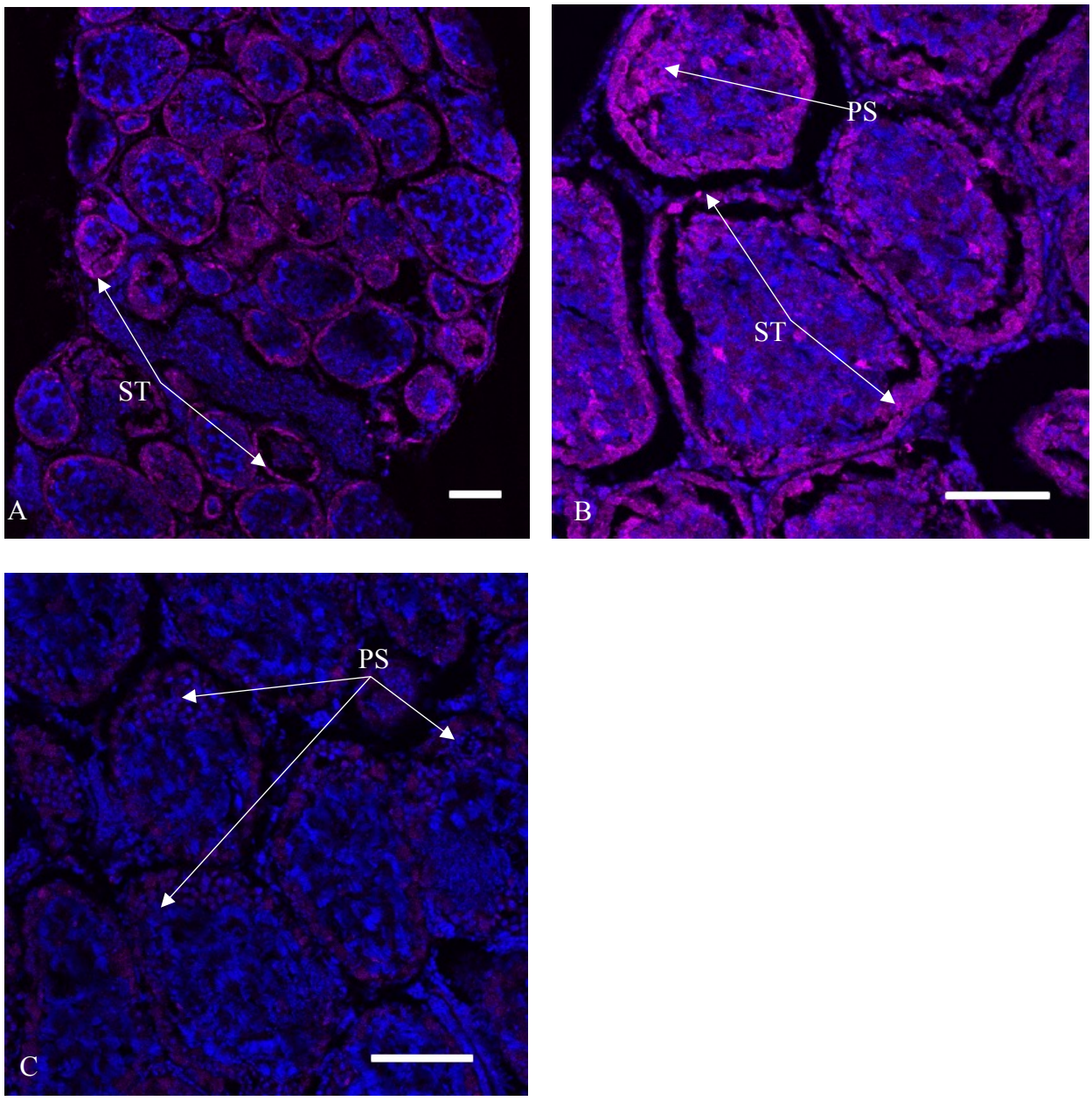

Figure 8. Immunohistochemistry of (A) cold-acclimated, (B) frozen, and (C) thawed testes for HC-7. Signaling is localized to seminiferous tubules (ST) and primary spermatocytes (PS). Panel (A) is a 10X objective; Panels (B) and (C) are 20X. All scale bars represent 100μM.

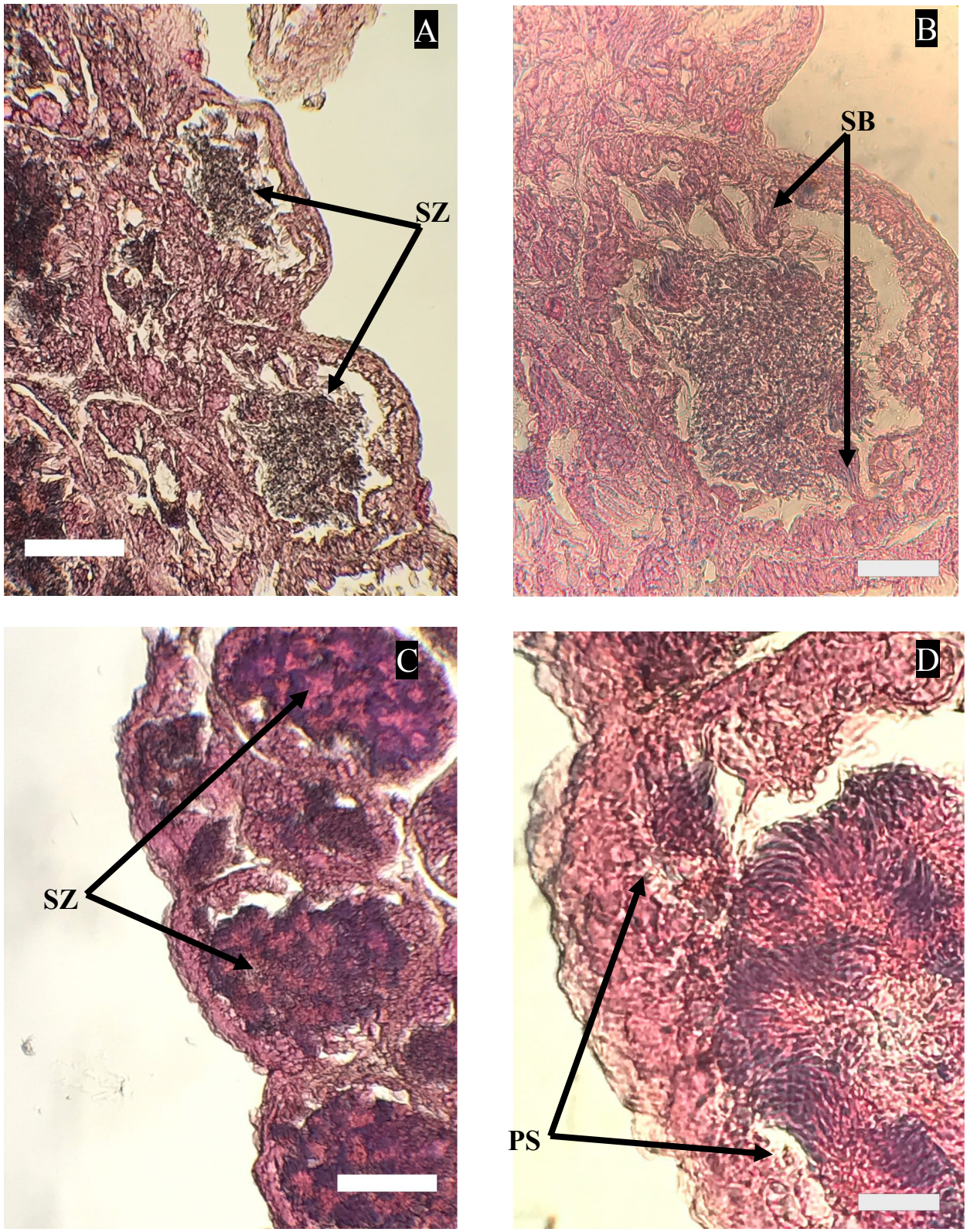

Figure 9A-H. Hematoxylin and eosin stains of warm (A-B), cold (C-D), frozen (E-F), and thawed (G-H) testes. Panels A, C, E, and G are at 10X magnification with a scale bar of 100µM; panels B, D, F, and H are at 20X magnification with a scale bar of 20µM.

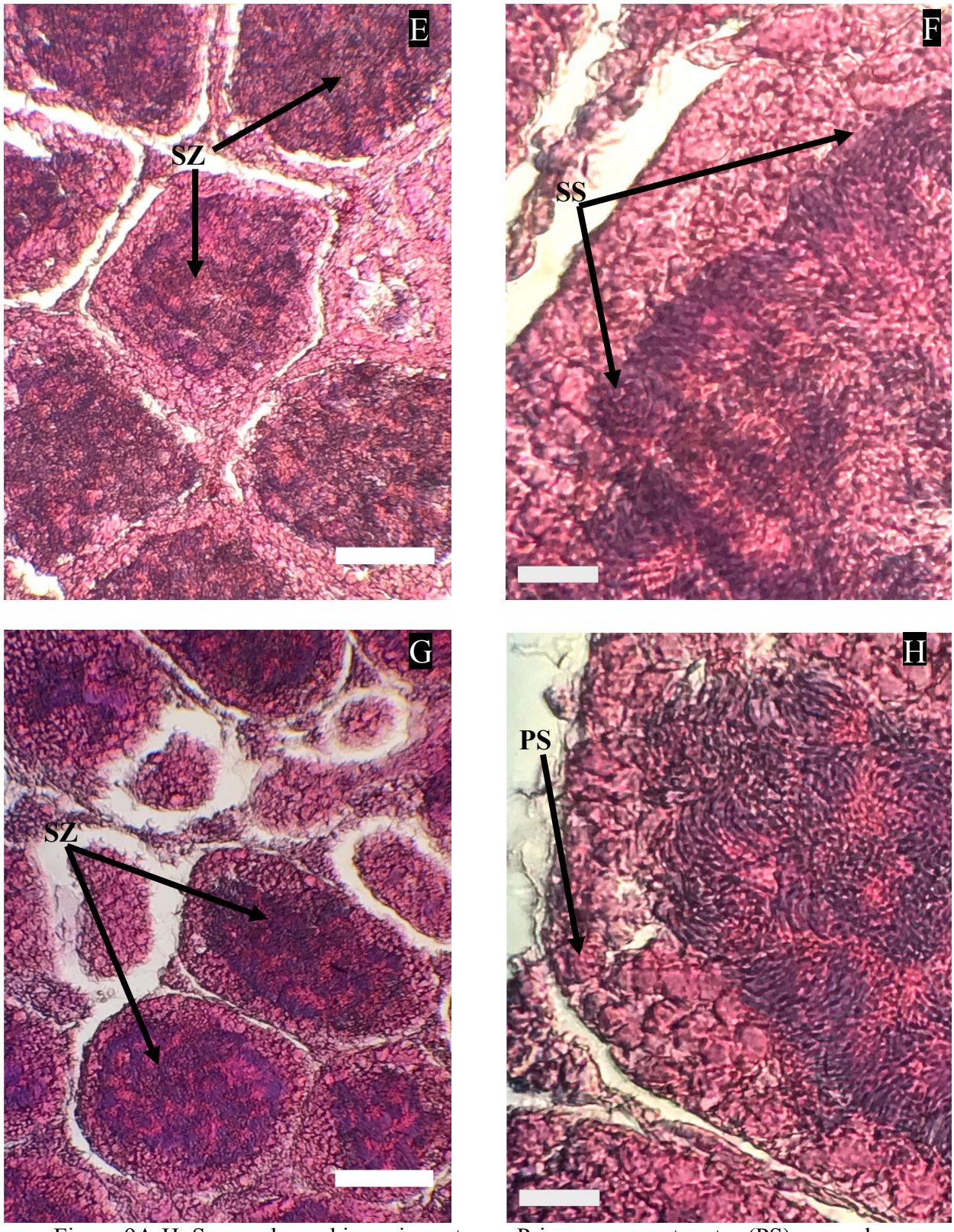

Figure 9A-H. Sperm showed in various stages. Primary spermatocytes (PS), secondary spermatocytes (SS), Spermatozoa (SZ), and sperm bundles (SB).

IV. Discussion

Dryophytes chrysoscelis exhibit a potentially continuous spermatogenic cycle

Amphibian spermatogenic cycles are dependent upon hormonal and environmental stimuli, and are categorized as discontinuous, potentially continuous, and continuous. Previously, the spermatogenic cycle in the gray treefrog *Dryophytes chrysoscelis* has not been identified. These frogs were responsive to intraperitoneal injections of human chorionic gonadotropin. Moreover, in our study, captive, warm-acclimated animals maintained with constant temperature (20°C) and light cycle (12hour light:12hour dark) remained responsive to hCG long after the end of their natural breeding season, even into the winter months. hCG is thought to act in one of two ways in stimulating sperm release. Neuronal excitability in the hippocampus may be modified by hCG injections, which stimulates downstream behaviors such as mature sperm release. Receptors in the testes may also bind to hCG and initiate steroid production and spermiation (Kouba et. Al 2012). Discontinuous spermatogenic cycles are characterized by having a distinct breeding season, and possessing the inability to be hormonally or environmentally stimulated outside of breeding season to produce or release sperm. Discontinuous breeders also show a lack of sperm at any stage within the testes during their off season (Rastogi *et al.* 2011). Our findings suggest that the Cope's gray treefrog does not exhibit discontinuous breeding due to its ability to be hormonally stimulated outside of breeding season. Moreover, these data suggest that the Cope's gray treefrog has a potentially continuous spermatogenic cycle as defined by the ability to be hormonally or environmentally stimulated to produce and release sperm outside of its defined breeding season (Rastogi *et al.* 2011). Further support for this conclusion comes from hematoxylin and eosin staining of the testes from cold, frozen, and thawed frogs, which contain sperm cells in various stages within the seminiferous tubules. Because of this type of

spermatogenic cycle, we could collect sperm samples year-round to assay for motility and aquaporin expression.

Activation of sperm motility is dependent upon osmolarity, not temperature

The effects of osmolarity on sperm motility activation have been studied in saltwater fish such as the marine gilthead seabream *Sparus aurata* (Boj, Chauvigné & Cerdà 2015), and in the ground-dwelling Australian frog *Crinia signifera* (Byrne *et al.* 2015). Sperm from the gilthead seabream were inactivated in NAM and when incubated with 0.3μM antibodies for Aqp-1aa, 1ab, and 7ab and subsequently immersed in saltwater for 3 minutes. Moreover, incubation with $HgCl_2$, which would likely inhibit all aquaporins, reduced the total percentage of motility. Compared with cells suspended in NAM, sperm in saltwater had a 3-fold increase of intracellular calcium concentrations (Boj, Chauvigné, and Cerdà 2015). Blockade of Aqp-1aa partially reduced the increase of calcium concentration, thereby reducing total motility. These data indicate that an increase in $[Ca^{2+}]$, induced by osmotic shrinkage via Aqp-1aa, is necessary for motility activation.

Medium osmolality significantly influenced sperm activation in the ground-dwelling Australian frog. Sperm were inactivated in NAM and exposed to hypoosmotic solutions of 200mOsm kg^{-1}, 100 mOsm kg^{-1}, 75 mOsm kg^{-1}, 50 mOsm kg^{-1}, 25 mOsm kg^{-1}, and 10 mOsm kg^{-1}. Motility reactivation was significantly greater at the lowest three osmolalities, and decreased as osmolality increased (Byrne *et al.* 2015). However, some motility was regained at all hypoosmotic dilutions.

Urine and seminal fluid osmolarities were measured from fresh collections. Prior to being injected with hCG, urine was collected from the cloaca, and had an osmolarity of 90.2±31.84

mmol/kg. Following an injection, osmolarity of seminal fluid containing sperm was measured at 60.09±10.82 mmol/kg. In *D. chrysoscelis*, as in those other species, motile sperm were immobilized by immersion in a ~290mOsm/L isosmotic environment. These cells were then able to regain motility when a hypoosmotic (200mOsm/L, 100mOsm/L, 75mOsm/L, 50mOsm/L, 25mOsm/L, 10mOsm/L) environment was reintroduced; the percentage of motility regained increased as osmolarity decreased to a peak at 50 mOsm/L. However, in contrast to the findings for *C. signifera*, a decrease in motility was seen in response to osmolarities below 50 mOsm/L. These data suggest that while a hypoosmotic environment will initiate motility, there is an optimal condition around 50 mOsm/L in which activation will occur. In both *D. chrysoscelis* and the ground-dwelling Australian frog, gametes are deposited into freshwater in nature. It seems likely that the osmotic environment in nature is substantially hypoosmotic, influenced by both the osmolarity of the freshwater environment and by the environment of the eggs deposited by the female. Therefore, peak activity at lower osmolarities is ideal for reproduction in nature. Sperm from both freshwater frog species, and from the marine gilthead seabream were successfully able to be inactivated in NAM, and reactivated when exposed to hypoosmotic diluents or saltwater respectively. This suggests that motility activation is dependent upon an intracellular change in concentration of salts such as calcium, or potentially by signaling mechanisms related to cell volume.

Our data suggests an optimal osmolarity for forward moving and circular sperm, but not for sperm who move in a wobble pattern or who are tail movers. As seen with total motility reactivation, a peak at around 50 mOsm/L is shown in forward moving sperm and circular movers. While it is plausible to think that forward projection is essential for egg fertilization, it is not yet known if egg penetration is dependent upon this motility pattern.

Optimal conditions for sperm activation were seen with osmolarity, however, we did not detect a similar finding for temperature. Sperm incubated at room temperature and 4°C showed no significant difference in their ability to regain motility. At both temperatures, sperm cells showed peak activity around 25 mOsm/L. The difference in optimal osmolarity in our two studies (50 vs. 25mOsm/L) may reflect some consequence of the duration that elapsed between sample collection and testing.

Sperm survive longer at cooler temperatures

Sperm viability was defined as the presence of motility, not by a live/dead assay. Viable sperm cells were ones that demonstrated any form of motility. These cells were able to maintain some degree of viability for a 24-hour period. Data from the Peron's treefrog, *Litoria peronii,* suggest that samples survive longer in cooler temperatures. Maximum sperm longevity in cooler temperatures ranged from 80h to 320h within the sample. The duration for motility to decline to 50% ranged from 25h to 125h at 4°C. In contrast, sperm from *L. peronii* stored at 23°C decreased to ~75% total motility after only 2 hours (Sherman *et al.* 2008). Our data suggest that sperm from *D. chrysoscelis* maintain viability more robustly at room temperature (>80% viability after 8 hours at 20) than do sperm from *L. peronii.* In both species, cooler temperatures induced a significantly prolongation of viability. For both species, the choice of habitat and the timing of reproduction clearly has a significant influence on the longevity of sperm after they are deposited.

No differences were detected between samples collected from the container versus those collected from the cloaca. The observed cells are in a motile state which suggests they were released in a hypoosmotic fluid; cells were not placed in any solutions for viability assays. In a

hypoosmotic solution, cells should experience swelling as water continues to influx which can eventually cause cellular lysing. This suggests that sperm cells employ a structural or internal biological process to prevent this phenomenon.

Presence of HC-1 in reproductive tissues

In all vertebrate species that have been examined, reproductive cells and tissues express aquaporins. In the marine teleost *S. aurata,* sperm express AQP-1-like proteins identified as AQP-1aa and -1ab (Chauvigné *et al.* 2013, Zilli *et al.* 2009). In White Koluda geese, AQP-1 was detected within the capillary endothelium of the testes and vas deferens (Skowronski *et al.* 2009). In the tropical bat, *Artibeus lituratus,* AQP-1 showed immunoreactions in testicular endothelia and in differentiating spermatids within the seminiferous tubules (Oliveira *et al.* 2013). HC-1 is ubiquitously expressed in previously tested tissues within *D. chrysoscelis,* though not every cell type in every tissue expresses the protein (Zimmerman 2007). Based on these previous findings, we hypothesized that aquaporin-1 would be expressed in the testes and mature sperm of Cope's gray treefrog. Consistent with our predictions, HC-1 was confirmed at the mRNA level in testes and, as a positive control, liver. However, no expression of HC-1 in sperm was detected. Due to testes containing mature sperm cells, the lack of expression in ejaculated sperm suggests that HC-1 is truly a testicular or perhaps an immature sperm protein, but is not a spermatozoa protein. Sperm, liver, and warm testes samples were run with primers for β-actin to ensure cDNA quality. Due to expression of β-actin at the mRNA level in sperm, we can conclude that the lack of HC-1 detection was not due to protocol error. Therefore, we accept that at the mRNA level, the HC-1 sequence was not present. However, the gilthead seabream showed expression of Aqp-1 like

proteins Aqp-1aa and Aqp-1ab. It is plausible that aquaporin-1 is present within the released sperm cells in a different isoform that is not specific to our current primers.

HC-1 was also analyzed at the protein level via SDS-PAGE and western blotting, and immunohistochemistry for localization. Western blots against HC-1 showed signaling in liver, and testes of warm, cold, and thawed conditions. Two bands were seen in testicular samples, suggesting a modified, potentially glycosylated, form of the protein. These findings are consistent with the mRNA findings. Also, consistent with those findings, we were unable to detect HC-1 protein in sperm. Probing with β-actin to ensure quality protein extraction showed signaling for tissues, but not sperm. Because PCR amplification was achieved for β-actin in sperm, this lack of signal in the Western blot indicated an error in protein extraction. A ponceau S stain was done to detect any level of protein for what should have been 10μg, 20μg, 30μg, and 40μg of sperm sample, and 10μg of liver sample. No protein was detected in any sperm sample (N=4). These calculated amounts were derived from the BCA assay, in which sperm and tissues were analyzed together. A color change was observed in the cuvettes containing sperm protein following the 30-minute incubation at 37°C, which was seen in tissues as well. The spectrophotometer gave an absorbance for sperm samples that fell within the standards 0μg/μl, 2.5μg/μl, or 5μg/μl on all attempts. Therefore, protein was assumed to be in the sample. False positive BCA readings could be due to the sample containing lipids or lipoproteins (ThermoScientific troubleshooting for BCA). 2% SDS was added to the sample to eliminate lipid interference. However, the spectrophotometer readings did not change. Sperm samples were treated with pipette mixing, glass homogenizer, and electronic homogenizer to attempt to disrupt cell membrane, but no protein was detected by either of those methods. Sperm samples were also boiled at 100°C in 2X Laemmli buffer, and no protein was detected by Ponceau S staining.

Immunolabeling of HC-1 was done in ejaculated sperm and testes from warm-acclimated animals that contained mature sperm. The ejaculated sperm smeared onto a slide did not show presence of HC-1 in the head or tail; this does not support the hypothesis but is consistent with mRNA expression. Cross sections of warm testes showed expression HC-1 in the mesorchium and in interstitial Leydig cells. DAPI nuclear stain was clearly able to detect presence of mature sperm within each tubule, but no expression of HC-1 was seen in the spermatozoa. HC-1 also was not detected in immature sperm cells. These data suggest that HC-1 is not expressed in any stage of sperm, but is a testicular protein. However, due to the inability to extract protein from sperm, it cannot be concluded if HC-1 is or is not present in sperm cells, although the null results with immunohistochemistry of sperm and testes suggests that HC-1 is not a sperm protein. The interstitial cells, or Leydig cells, where HC-1 was expressed, function to produce testosterone, a hormone required for spermatogenesis. This suggests that aquaporin expression could be a factor in testosterone production, and ultimately for spermatogenesis.

HC-7 is present among tissues and immature sperm cells

Like Aqp-1, Aqp-7 expression was detected at the mRNA level in *S. aurata* (Boj 2015). Likewise, AQP-7 has been found in sperm and testes in species ranging from marine teleosts (gilthead seabream: Boj 2015) to birds (White Koluda geese; Skowronski 2009) and mammals (rats: Chen and Duan 2011). Because of this, we hypothesized that Aqp-7 would also be present in anuran sperm and testes. PCR analysis for HC-7 primers yielded similar results as seen in HC-1. PCR product was detected for liver and testicular tissues, but not for sperm cells. As also seen in HC-1, the data suggests that HC-7 is a testicular or immature sperm protein, but not a mature sperm cell protein.

Protein analysis of HC-7 showed signal in liver and warm, cold, and thawed testes, but did not show signal in sperm. Testes samples did not show a potentially glycosylated form of the protein. Liver did show a single band at around 43 kDa which suggests a modified form; the expected size of HC-7 is 31.7 kDa. Expression in testes and liver but not in sperm indicate that HC-7 is not a sperm protein. However, due to the lack of successful protein extraction, that could be determined directly.

Immunolabeling of warm testes shows signaling in the interstitial tissues, as well as primary spermatocytes. Unlike HC-1, in the Leydig cells, the spatial pattern for HC-7 in the Leydig cells was not localized to specific cells. Rather, HC-7 immunolabeling appeared throughout the interstitial tissue and seminiferous tubules. The interstitial cells are thought to have some endocrine function (Rugh 1951) and the seminiferous tubules are the site for spermatogenesis, which suggests that HC-7 may have a role in stimulation of spermatogenesis. Immunolabeling of HC-7 can also be seen in primary spermatocytes, but not in mature sperm bundles or spermatozoa within the testes. These data suggest that HC-7 is necessary for early stages of sperm maturation, but may not be necessary in latter stages. HC-7 is present in the testes and liver but could not be detected in mature sperm within the lumen. HC-7 is a part of the glyceroporin family. As seen in the IHC images, HC-7 remains in the interstitial tissues and primary spermatocytes through the freezing process. In Cope's gray treefrog glyceroporins transport water out of the cells and glycerol in as a cryoprotectant during winter months. It is possible that HC-7 not only aids in stimulation of spermatogenesis, but also protects the testicular tissues from cryoinjury during winter.

V. Conclusion

Our work has demonstrated that activation of sperm motility is largely dependent upon environmental osmolarity. Frogs are external fertilizers and deposit their gametes into freshwater. Therefore, sperm must function in an environment that is hypoosmotic relative to the testes. Sperm cells must traverse an osmotic gradient beginning in an isosmotic fluid within the testes. Upon release, seminal fluid containing sperm potentially mix with hypoosmotic urine, which immediately activate motility; these activated cells are then deposited into the freshwater ponds. Our data have shown that a more hypoosmotic environment increases the percentage of total motile cells. However, our data suggest that there is an optimal range with the peak value being 50mOsm/L. These data show that sperm have an optimal functionality when experiencing a shift in osmolarity which is ideal for their mode of reproduction.

Temperatures may fluctuate during the breeding season, which could potentially affect sperm viability. Our worked explored the effects of temperature on motility activation and viability, and found that sperm may continue to function properly independent of temperature. When comparing cooler temperatures to warmer temperatures, there was no difference in the percentage of cells that regained motility. While cells maintained at 4°C had a higher viable percentage after 24 hours, cells held at room temperature showed a high percentage of total motility after 12 hours. In nature, sperm is directly deposited onto the egg cells. This would indicate that fertilization occurs relatively quickly. However, sperm deposited into warmer waters can maintain its viability for 12-24 hours post release.

When experiencing a strong hypoosmotic shock, cells tend to swell until they burst. While our study did not assess cell lysing, we did see that a large percentage of motile cells maintained their integrity in hypoosmotic urine and seminal fluid after 24 hours; this would indicate that

these cells did not lyse. However, it is known that a change in intracellular volume or concentrations must occur for motility to activate. Therefore, we examined the expression of aquaporins in the sperm cells. Our data shows that at the mRNA level, neither HC-1 or HC-7 were present in the released sperm cells. Immunohistochemistry likewise suggests that these two aquaporin proteins are expressed in testicular cells but not in mature sperm. These two proteins were explored because they appeared to be the most ubiquitous sperm aquaporins among vertebrates. However, it is possible that a combination of the other aquaporins are present within these cells. It is also possible that while there are no mRNA transcripts in the cells, the protein is there but was undetectable by our methods. We predict that aquaporins other than HC-1 and HC-7 function within the sperm of Cope's gray treefrogs to transport water into the cell and initiate motility.

Further impacts

Our work has supported the theory that sperm activation is dependent upon osmotic shocks, but is not temperature dependent. We have shown that sperm can be successfully inactivated in an isosomotic environment as would exist within the testes. We then were able to reactivate the cells in a series of hypoosmotic solutions. However, we did not identify the intracellular ionic concentrations that enable motility. Previous studies have looked at solutes such as intracellular calcium and potassium in active versus inactive sperm (Boj, Chauvigné and Cerdà 2015; Morisawa *et al.* 1983). A study conducted in *Xenopus laevis* also examined the effects of pH on sperm motility (Christensen *et al.* 2004).

The determination of aquaporin expression in the sperm cells should also be further tested. Our study focused on HC-1 and HC-7. The other aquaporins and glyceroporins could be

assessed at the mRNA and protein level. It would also be worthwhile to find a protocol that could achieve a proper protein isolation from sperm cells on a small sample. Further exploration on the roles of aquaporins in both the sperm (immature and mature) and the testes could be conducted. Our data suggests proteins from the aquaporin family could have roles in spermatogenesis or in cryoprotection, but those have not been specifically studied. By blocking aquaporins in the sperm or testes, we could better identify the role of these aquaporins.

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