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Development of cryopreservation methods for sperm of pinto abalone (Haliotis kamtschatkana) for conservation aquaculture.

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Development of cryopreservation methods for sperm of pinto abalone, *Haliotis kamtschatkana*, for conservation aquaculture.

By
Caitlin O'Brien

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

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Master's Thesis

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Caitlin O'Brien
May 21st, 2019

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A Thesis Presented
to The Faculty of
Western Washington University

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ABSTRACT

The pinto abalone, *Haliotis kamtschatkana*, is an ecosystem engineer with cultural and ecological significance. Due to the dramatic 98% decline in populations, a ten-year recovery plan was enacted to bring levels of pinto abalone back from the brink of extirpation in the San Juan Archipelago, Washington. The focus of this study was to assist restoration efforts in creating self-sustaining populations by developing methods of cryopreservation for male pinto abalone sperm. My study evaluated three commonly used cryoprotectants, a series of freeze/thaw temperatures, and developed methods of quality assessment specific to pinto abalone. Animals were provided and cared for by Puget Sound Restoration Fund and all experiments were done at the NOAA Research Station in Manchester, WA.

I evaluated sperm quality with a computer-assisted sperm analysis (CASA) system, for which I developed parameters to track pinto abalone sperm motility. I used sperm motility to analyze sperm quality in a series of experiments culminating in a final experiment to test the optimized cryopreservation methods by fertilizing pinto abalone eggs. Toxic effects of commonly used cryoprotectants, di-methyl sulfoxide (DMSO), glycerol (GLY), and propylene glycol (PG), were tested at concentrations from 5%-20%. DMSO at 5% was the least toxic, yielding the highest percent motile sperm after an exposure time of 10 minutes. Using a programmable freezer, a series of freezing rates and endpoint temperatures were evaluated for cryopreservation of sperm in 0.5 mL freezing straws at a density of 1.8×10^8 sperm mL^{-1}. A freezing rate of -3 ℃ min^{-1} to an endpoint temperature of -60 ℃, preservation in liquid nitrogen (-196℃), then thawing in a 40℃ water-bath for 8 seconds yielded the highest sperm motility. Cryopreserved sperm successfully fertilized eggs, but with lower success than with untreated sperm. The highest percent fertilized was 12.2% using a concentration of 1×10^6 sperm mL^{-1} with 14.2% post-thaw motility. Through this study, I have outlined the first attempt into cryopreservation of pinto abalone sperm and provided a foundation for future research to optimize the methods developed. Developing cryopreservation methods will allow hatchery managers to build a genetic library to improve production and maintain genetic diversity of this vanishing species.

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INTRODUCTION

The pinto or northern abalone, *Haliotis kamtschatkana*, is in severe decline in Washington State waters. Abalone are ecological engineers and key members of the rocky subtidal community (Rogers-Bennett et al. 2011; Sloan & Breen, 1988). Continual grazing of micro- and macro- algae by this marine gastropod clears habitat for new species recruitment and nutrient cycling. Abalone were never commercially harvested in Washington State and all recreational harvest was closed in 1994. However, significant levels of legal and illegal catch in the 1980s and early 1990s were possible contributors to the 98% decline in populations from 1992-2017 (Bouma et al. 2012; Rothaus et al. 2008; Washington Department of Fish and Wildlife [WDFW] 2014). Abalone are dioecious broadcast spawners that aggregate during reproduction and require a minimum density of 0.15-0.3 individuals m^{-2} to successfully reproduce (Babcock & Keesing, 1999; WDFW, 2014). Based on surveys conducted in the San Juan Archipelago since 1992, it is likely pinto abalone populations have been under this minimum density for more than 20 years and are no longer successfully reproducing in the wild (Rothaus et al. 2008). Due to the pinto abalone's inability to reproduce in the wild, populations will not likely rebound without human intervention.

In an effort to forestall complete stock collapse, Puget Sound Restoration Fund (PSRF) and Washington Department of Fish and Wildlife (WDFW) have collaborated to rebuild natural populations of pinto abalone in the Puget Sound through responsible conservation aquaculture. Conservation aquaculture is predicated on the principle that genetic diversity must be maintained to limit deleterious traits being introduced into natural populations. To meet this fundamental requirement, the Pinto Abalone Recovery Plan uses only single-parent crosses of wild-caught broodstock to produce first-generation progeny for restoration sites in the San Juan Archipelago. Based on this design, from 2004-2017 PSRF and WDFW outplanted 15,000 hatchery-reared pinto abalone to 12 restoration sites in the San Juan Archipelago (Carson et al.2019; WDFW & PSRF *personal communication*, 2018). However, between

1989-1990 self-reported recreational harvest levels were around 40,000 individuals per year, with a high likelihood of underestimation due to the lack of reporting (Neuman et al. 2018; WDFW, 2014). While the restoration project has undeniably made progress, total abalone outplanted over 13 years has yet to exceed the take that occurred historically in a single year of abalone harvest (Carson et al. 2019).

Production of genetically diverse progeny is a limitation to reaching minimum density thresholds at restoration sites. Hatchery spawning events are limited by personnel, available hatchery space, production cost, and time constraints due to gamete degradation. Hatchery spawning events can be stressful for abalone since induction of spawning requires exposure to higher than normal pH seawater and temperature fluctuations. Therefore, it is common practice to avoid repeatedly using the same individuals in a spawning season to reduce the risk of mortality (Babcock & Keesing, 1999; WDFW & PSRF *personal communication*, 2018). In the highly reduced population that exists in Puget Sound, replenishing wild-caught broodstock becomes more difficult with each passing year. In addition, during a hatchery spawning event it is unlikely that all animals selected will produce viable gametes, thereby limiting available single-parent cross options and greatly decreasing the genetic diversity of progeny. Occasionally, only one sex produces gametes, or, alternatively, no new crosses can be formed with the animals that do spawn. This results in wastage of sperm and eggs during a spawning event. With so much care, time, and cost going into producing pinto abalone, there is little room for inefficient practices such as wasted gametes during a short breeding season (late May to early October) (Sloan & Breen, 1988; Stekoll & Shirley, 1993). One solution to improving production, while maintaining genetic diversity, is to develop a way to preserve excess gametes for future use.

Cryopreservation allows for the preservation of gametes by exposing cells to extremely low temperatures without causing damage or rupture. Specialized chemicals, called cryoprotectants, add macromolecules that reduce the formation of damaging ice crystals on the cell membrane during the freezing process (Liu et al. 2015a). Preserved gametes can later be thawed and used for fertilization,

thereby extending the very short shelf-life gametes typically have (Babcock & Keesing, 1999; Baker & Tyler, 2001). Cryopreservation has been used in many marine molluscs with increasing success as methods are refined, but cryopreservation techniques must be tailored to each species for the best results (Liu et al. 2015a; Liu et al. 2014b; Salinas-Flores et al. 2005). No protocol has been developed specifically for pinto abalone. Thus, a cryopreservation protocol for pinto abalone is needed to allow the Pinto Abalone Recovery Project to maintain a genetic library of individual gametes to supplement spawning events, allowing broodstock to be returned to their native habitat to continue to contribute to the gene pool, while also freeing up much needed hatchery space and limiting production costs.

The goal of my study was to develop the first protocol of cryopreservation methods for pinto abalone sperm. My research objectives were to: 1) determine which of three widely used cryoprotectant types, concentrations, and exposure times has the least toxic effect on pinto abalone sperm, 2) determine a combination of freezing rate, endpoint temperature, and thawing temperature using a programmable freezer that maximizes success of cryopreservation, and 3) develop methods for evaluation of cryopreserved sperm viability as measured by percent motility and fertilization.

METHODS

Experimental Overview

I conducted experiments testing cryoprotectant type, concentration, and exposure time to determine the combination that caused the lowest acute toxicity to pinto abalone sperm. I then used this cryoprotectant combination to test freezing rates, endpoint temperatures, and thawing temperatures using a programmable freezer. For each of these experiments, I conducted range-finding preliminary experiments on filial generation of pinto abalone to inform the final ranges used in experiments with wild-caught broodstock pinto abalone. I used sperm motility, assessed via a computer tracking program, to evaluate sperm quality post-treatment for the experiments described above. I then used a final fertilization experiment to evaluate the effectiveness of the cryopreservation techniques developed in the earlier experiments. Figure 1 illustrates the sequence of experiments.

Animal and gamete collection

I completed all experiments at the Manchester NOAA Research Station in Manchester, Washington in collaboration with Puget Sound Restoration Fund (PSRF), Washington Department of Fish and Wildlife (WDFW), NOAA National Marine Fisheries Service, and Western Washington University (WWU). Adult wild pinto abalone were provided and cared for by PSRF in partnership with WDFW. Filial generations of broodstock animals were used for preliminary experiments and wild broodstock were used for final experimental trials. Animal selection for experiments was based on ripeness of gonad following the criteria used by PSRF (Table 1). Only animals with a gonadal index greater than one were used in this study. Ages of wild animals were unknown, however the size range was 115-146 mm with weights of 231.3-555.1 g, indicating mature animals (Neuman et al. 2018). Animals were spawned following the PSRF spawning protocol developed and tested specifically for pinto abalone (WDFW & PSRF *personal communication*, 2018). Per this protocol, all seawater was UV-irradiated and filtered to 5 µm. Fecund

abalone were placed into individual buckets with 7 L of aerated seawater at 14 ℃ (temperature of holding tanks) to isolate gametes. The buckets were then placed in a water bath at 17 ℃ (or 3 ℃ warmer water than holding tanks) to allow acclimation to spawning temperatures. To induce spawning, abalone were next exposed to seawater buffered to pH 9.1 using 2 M Tris buffer solution and 6% H_2O_2 per 1 L of seawater in the 7 L holding buckets. Abalone were left in solution for up to three hours, or until spawning commenced, then were rinsed and placed in fresh seawater to remove trace chemicals in the buckets.

Once spawning began, sperm was collected by direct pipetting from the abalone's exhalent respiratory pores. All experiments were conducted in a walk-in freezer set to 10℃ and were completed under 3 hours from the time of sperm release. Concentrated sperm was kept at 10 ℃, similar to ambient seawater temperatures, until all experiments were completed. Sperm concentrations were determined using a hemocytometer to count sperm diluted with 0.2 µm filtered seawater at a ratio of 1:20.

Sperm motility assessment

I used Computer Assisted Sperm Analysis (CASA) to track sperm movement in video recordings to identify and count the percent motile versus non-motile sperm. CASA was developed as a way to track and analyze sperm movement in a reliable, reproducible, and more accurate method than subjective observation (Acosta-Salmón et al. 2007; Hassan et al. 2014; Liu et al. 2015a). The CASA system allows for high throughput of videos to quantify sperm motility with definitive criteria. The three main categories from which percent motile is calculated are velocity curvilinear (VCL), linearity (LIN), and bulk flow of sperm. Velocity curvilinear is the speed of sperm from point to point, whereas linearity encompasses the overall speed and straightness of a sperm's path. Parameters for bulk flow are included to discern true sperm motility versus movement due to fluid flow. If the minimum thresholds are met for VCL and LIN, and no movement due to bulk flow is detected, then sperm are considered motile and are represented in the final percent motile output expressed in my results.

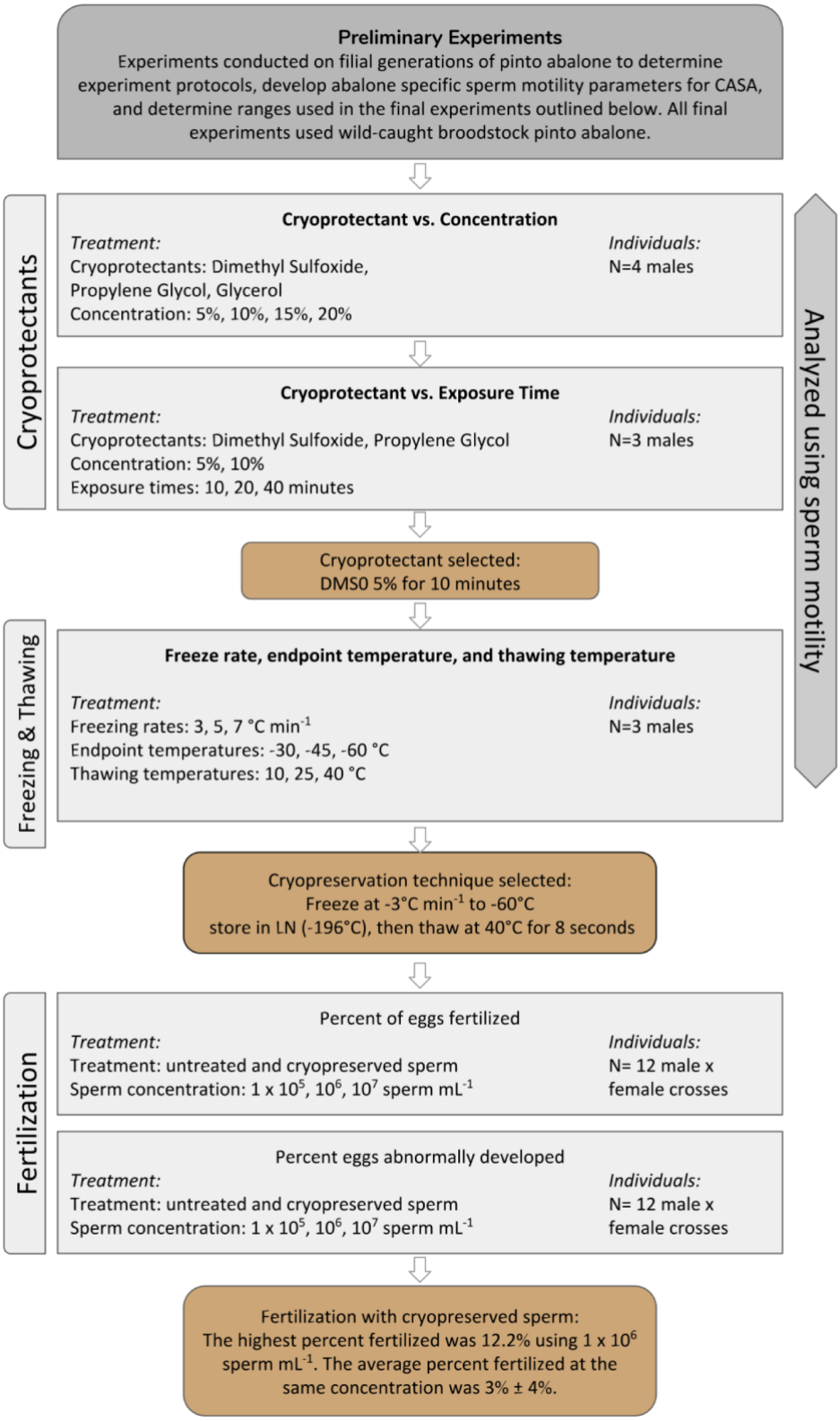

Figure 1. *Flow chart of experimental design* | Flow chart highlights each final experiment conducted on wild-caught pinto abalone broodstock used to develop methods of cryopreservation.

Table 1. *Gonad Selection Criteria per PSRF Protocol* | Animal selection for experiments was based on ripeness of gonad following protocol developed by PSRF (WDFW & PSRF *personal communication*, 2018). Only animals with a gonadal index greater than one were used in this study.

Scale	Gonad Index
0	No development of gonad, sex indeterminate.
1	Gonad beginning to show signs of swelling and gamete production. Male gonad beige to orange in color and female gonad green to purple in color.
2	Gonad swelling up to level of shell margin, partially mature, may spawn.
3	Gonad swelling above level of shell margin, fully mature and ready to spawn.

Table 2 describes a condensed version of the parameters, excluding bulk flow parameters, that are explained fully in Wilson-Leedy & Ingermann (2011). To use CASA for abalone, the minimum parameters based on fish sperm were updated to reflect sperm movement for pinto abalone using MTrackJ (Meijering et al. 2012). MTrackJ is a manual tracking computer program that allows the user to manually select the track of individual sperm from one frame to the next in a video recording. By manually tracking individual sperm movement, I was able to determine the general size, speed, length of track, and straightness of path associated with pinto abalone sperm. Once determined, I repeatedly updated CASA parameters to reflect pinto abalone sperm movement until I achieved a reliable automated computer tracker that could be visually confirmed by a computer-generated sperm pathway output (Figure 2). Table 2 shows CASA stock fish parameters versus the final pinto abalone parameters determined by manual tracking.

To collect video recordings, sperm concentrations were diluted to 10^4–10^6 sperm ml^{-1} using 0.2 µm filtered seawater based on CASA recommendation (Immerman & Goetz, 2014; Wilson-Leedy & Ingermann, 2011). Diluted sperm was loaded into 8-chambered (20 µm channel) Leja sperm analysis slides (Leja, Netherlands) for single planar video analysis. All videos were recorded using an Allied Prosilica Vision Model GC650 camera's stock magnification with darkfield microscopy utilizing a 4x objective (Olympus IM, Center Valley, PA). Videos were recorded at 90 frames per second for 10 seconds.

To analyze the videos, I only used frames 300 to 400 in each video to avoid the bulk flow of sperm resulting from the capillary fill of Leja slides (Lu et al. 2014). I used ImageJ FIJI version 1.52e auto-threshold with a variance filter to remove background gradation and highlight black sperm heads on white background for optimal tracking in CASA. I visually inspected each video for quality control by eliminating incorrect pathways due to debris, flow, or nearness of other sperm. Quality controlled videos were then tracked in ImageJ version 1.52a with a CASA plug-in to assess motile versus non-motile sperm (Figure 2).

Table 2. *Sperm Motility Parameters*| To be considered motile (MOT) using Computer Assisted Sperm Analysis (CASA), sperm must meet minimum velocity curvilinear (VCL) and linearity (LIN) parameters, in addition to parameters developed to detect bulk flow of sperm (CASA parameter metrics h-p). The table below show the updated pinto abalone parameters versus CASA's stock parameters used for analysis along with a definition box for VCL, LIN, and MOT. A full description of the parameters can be found in *Wilson-Leedy & Ingermann, (2011)*.

CASA parameter metrics:		CASA stock values	Abalone values*
a.	Minimum sperm size (pixels)	0.00	10.00
b.	Maximum sperm size (pixels)	40.00	300.00
c.	Minimum track length (frames)	97.00	30.00
d.	Maximum sperm velocity between frames (pixels)	8.00	8.00
e.	Minimum VSL for motile (µm/s)	3.00	10.00
f.	Minimum VAP for motile (µm/s)	20.00	50.00
g.	Minimum VCL for motile (µm/s)	25.00	50.00
h.	Low VAP speed (µm/s)	5.00	5.00
i.	Maximum percentage of path with zero VAP	1.00	1.00
j.	Maximum percentage of path with low VAP	25.00	25.00
k.	Low VAP speed 2 (µm/s)	25.00	25.00
l.	Low VCL speed (µm/s)	35.00	35.00
m.	High WOB (percent VAP/VCL)	80.00	80.00
n.	High LIN (percent VSL/VAP)	80.00	80.00
o.	High WOB two (percent VAP/VCL)	50.00	50.00
p.	High LIN two (percent VSL/VAP)	60.00	60.00
q.	Frame Rate (frames per second)	97.00	90.00
r.	Microns per 1000 pixels	1075.00	2000.00

* I developed pinto abalone parameters using an Allied Prosilica Vision Model GC650 camera with a 4x objective using darkfield microscopy.

Sperm motility parameter definitions:		
VCL	Velocity Curvilinear	Point to point velocity per second (µm/seconds)
LIN	Linearity	Straightness of path (0, curved to 1, straight), calculated by using VSL/VAP, both described independently below: VSL, Velocity Straight Line: Velocity measured from the first point to the furthest point of origin using an average path during a set time. VAP, Velocity Average Path: Point to point velocity using roaming averages (based on 1/6th the frames)
MOT	Percent Motile	Percent of sperm that meet the minimum VCL, VSL, and VAP set in the parameters above.

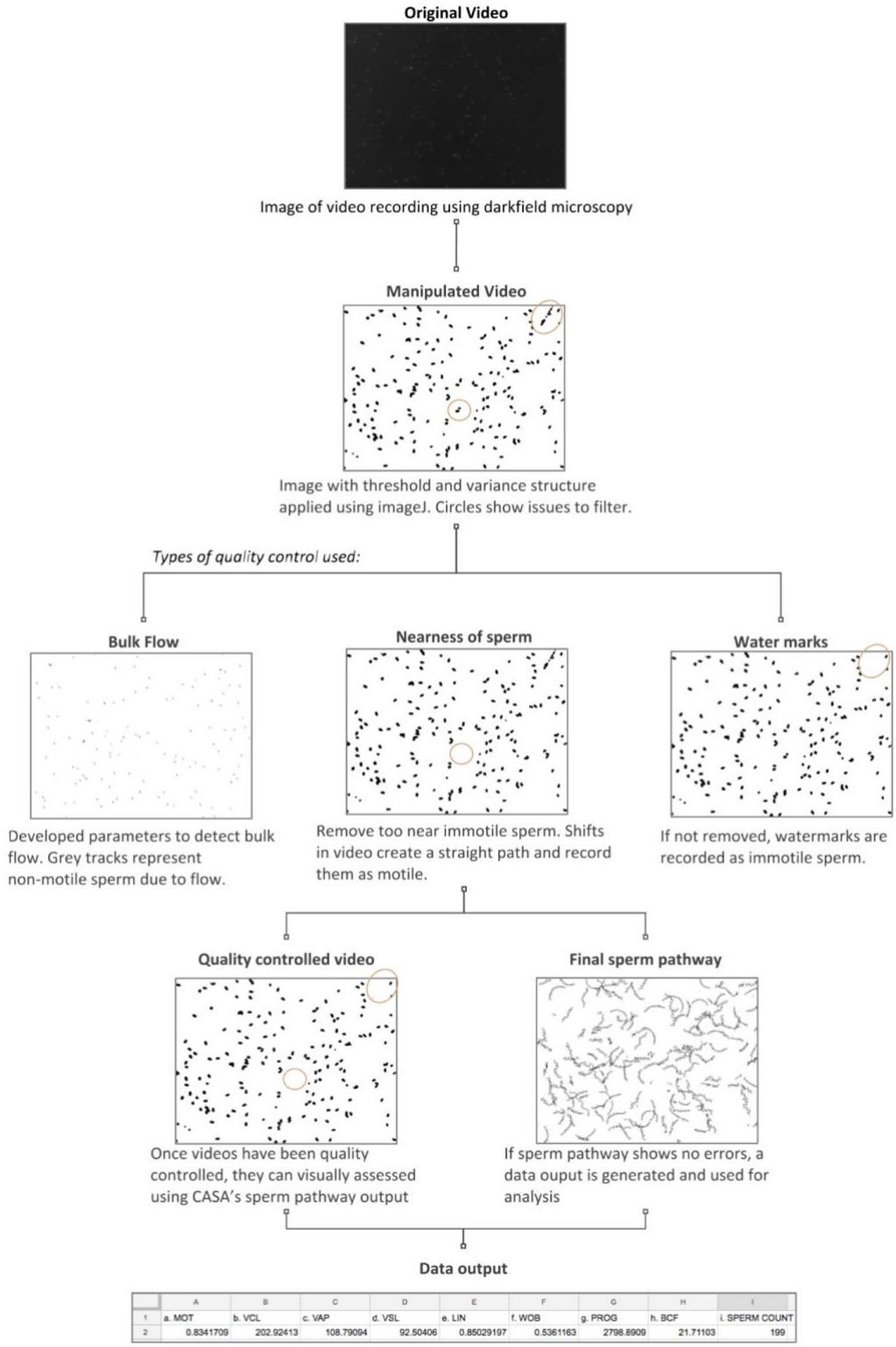

Figure 2. *Video recording manipulation and quality control* | The flowchart above shows the stages used to manipulate and quality control video recordings then used in the Computer Assisted Sperm Analysis (CASA) program to track motile versus non-motile sperm.

Statistical Analysis

I performed statistical analyses using R Studio package nlme (Pinheiro et al. 2018). Sperm motility was used to determine the least toxic cryoprotectant type, concentration, and exposure time, along with the least damaging freezing rate, endpoint temperature, and thawing temperature. Percent of eggs fertilized and percent of abnormally developed eggs were used in the final fertilization experiment. All results reported are untransformed values. The original data were used in a Generalized Linear Mixed Model (GLMM) analysis to allow for random factors such as individual abalone to be included within the experimental design. I used a top-down model fitting approach by including the full model with all fixed factors, random factors, and interactions, then choosing the most parsimonious model based on the lowest Akaike's Information Criterion (AIC) (Burnham & Anderson, 2002; Theobald, 2018). Corrected Akaike information criterion (AICc) were reported using R package AICmodavg to correct for small sample sizes (Mazerolle, 2017; Theobald, 2018). Fixed effects, interactions, and random effects were not included unless significant (α= 0.05). Weighted variance structures in the nlme package were applied to lessen heterogeneous variance associated with individual to individual variation by allowing for the variance to increase with the mean. Specific experimental design and the final statistical models used in the analyses are described in the sections below.

Experimental methodology

Cryoprotectants

I assessed the effectiveness of three commonly used cryoprotectants: di-methyl sulfoxide (DMSO), propylene glycol (PG), and glycerol (GLY). All cryoprotectants were AR grade purchased from Sigma-Aldrich Pty Ltd (St Louis, MO, USA). Percent concentrations were selected based on literature review with similar species (Liu et al. 2015a; Paredes, 2015) and preliminary experiments with filial generations of pinto abalone.

To assess acute toxicity of cryoprotectants on motility, I selected four male broodstock abalone and tested four concentrations (5%, 10%, 15% and 20%: v/v) of each cryoprotectant (DMSO, PG, GLY) and a control sample per male for a total of 52 samples (3 cryoprotectants x 4 concentrations x 4 males = 48 samples + 4 controls = 52 samples). The experiment was repeated for each male, in order of spawn time. Experimental treatments were randomly assigned across three six-well plates per male. Stock solutions of cryoprotectants were prepared using 0.2 µm filtered seawater at 2.5x the final concentration required in the experiment. Stock solutions were slowly mixed with sperm at a volume ratio of 1:1.5 to reach the final desired concentration. The final mixture was agitated on a stir plate for 10-13 minutes before motility was assessed.

In the final model, cryoprotectant type and concentration were included as fixed effects with no interactions. Cryoprotectant was modeled as a categorical factor and concentration as a continuous factor due to its linear relationship. Male was included as a random factor and a fixed weight using the nlme package was applied.

To determine acute toxicity from exposure time to cryoprotectants, I tested four different broodstock males at 10, 20, and 40 minutes of exposure to DMSO and PG at 5% and 10% concentration for a total of 13 samples per male (3 exposure times x 2 cryoprotectant x 2 concentrations x 3 males = 36 samples + 3 controls = 39 samples total). Only two cryoprotectants were used in this experiment since percent motility of sperm exposed to GLY was so low across all concentrations. Experimental treatments were randomly assigned across three six-well plates per male.

In the final model, cryoprotectant type and concentration were included as fixed effects with no interactions. Cryoprotectant was a categorical factor, whereas concentration was a continuous variable due to the linear relationships. Male was included as a random effect and a fixed weight was applied to male. Exposure time was not significant and was not kept in the final model. However, for subsequent experiments, exposure time selection was based on the highest percent motility.

Based on these acute toxicity tests, DMSO at 5% for 10 -13 min was used as the cryoprotectant type, concentration, and exposure time for the following freezing and thawing experiments.

Freezing and thawing

I ran a series of range-finding preliminary experiments with filial generations of pinto abalone to investigate optimal freezing rate, endpoint temperature, and thawing temperature. Treatment ranges used in preliminary experiments were based on supporting literature with similar abalone species (Liu et al. 2015a; Paredes, 2015). Following preliminary results, a final experiment investigated all three parameters interdependently using wild-caught broodstock.

Freezing of sperm was accomplished with a Crysalys Cryocontroller PTC-9500 Programmable Freezer (Biogenics, USA). Prior to freezing, sperm was loaded into 0.5 mL freezing straws (IMV technologies USA, Maple Grove, MN) at a concentration of 1.8 x 10^8 sperm ml^{-1}, similar to the concentration used by (Liu et al. 2014c) with *Haliotis laevigata*. The freezing straws were then placed in the programmable freezer and freezing rates of -3, -5, and -7 ℃ min^{-1} to endpoint temperatures of -30, -45, and -60 ℃ were applied to the samples. Experimental treatments were systematically assigned a position within the cryochamber to avoid chamber effects and were batched 18 straws at a time due to limited space within the chamber. Freezing straws were then thawed at room temperature (10 ℃), in a water bath at 25 ℃ for 12 seconds, or in a water bath at 40 ℃ for 8 seconds. The times associated with each temperature were determined in preliminary experiments and represent when the frozen straw turned from solid to liquid. Three broodstock males were used in the final experiment for a total of 54 samples per male (3 freezing rates x 3 endpoint temperatures x 3 thawing temperatures x 2 replicates per treatment = 54 samples x 3 males = 162 samples total).

In the most parsimonious model, endpoint temperature was included as fixed effects and no other fixed effects or interactions were included. Endpoint temperature was analyzed as continuous

variable. Male was included as a random effect and included a random intercept for each male with no weighted variance structure applied. Videos of sperm from each male were taken prior to treatment to ensure percent motility was above 70%. No control samples went through the freezing and thawing process therefore the percent motility of untreated males were not included in the analysis.

Freezing rate and thawing temperature were not significant and were not included in the final model. To select temperatures for the final fertilization experiment, I chose the highest mean percent motility for freezing rate and thawing temperature as fixed, continuous variables. Based on these results, a cryopreservation rate of -3 ℃ min^{-1} to an endpoint of -60 ℃ and thawing in a 40 ℃ water bath for 8 seconds were used in the final fertilization experiment.

Fertilization

To test fertilization success of cryopreserved sperm, three broodstock males were spawned, sperm was cryopreserved using methods described above and then stored in liquid nitrogen (-196 ℃) for one week prior to fertilization. On the day of the fertilization experiment, three females and one control male were spawned. Eggs from the three females were fertilized with cryopreserved sperm from the three experimental males and untreated sperm from the one control male for a total of 12 crosses using a full factorial matrix spawning design. I used a range of sperm concentrations to fertilize eggs based on the egg:sperm ratio used by PSRF and preliminary experiments with filial generations using treated sperm. During the preliminary tests, I experimented with sperm concentrations of 1 x 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 sperm mL^{-1}, and 1 x 10^5, 10^6, 10^7 sperm mL^{-1} yielded the highest fertilization success. Therefore, each male-female cross in this experiment was fertilized with these three sperm concentrations for 15 minutes in 10 mL of filtered seawater for a total of 36 samples (12 crosses x 3 concentrations = 36 samples). Egg concentration has been shown to be less important than sperm concentration in

determining successful fertilization, therefore eggs were held constant at 500 eggs per 10 mL well (Babcock & Keesing, 1999).

Treatments were assigned using a randomized design across six, six-well plates. Each well contained a 12.7 mm x 50.8 mm pvc filter with a 48 µm mesh attached to the bottom. This filter was used to hold eggs and allowed for a gentle and efficient transfer of eggs from one well-plate to the next during the rinsing process. After 15 minutes of exposure to sperm concentrations, the filters holding eggs were removed from wells, rinsed in 10 ml of filtered seawater, and transferred to new six-well plates with 10 ml of filtered seawater. Filtered seawater rinses were repeated each hour until fertilization scoring occurred after 4 hours. At this time, a sample of 100 eggs was placed in a Ward zooplankton counting wheel under a dissection scope to score fertilization. Eggs were deemed fertilized if the two-or-four cell stage had been reached. Percent fertilized was expressed as the number of eggs that successfully cleaved divided by the total number of eggs scored. A separate count was kept for abnormally developed eggs. Eggs were considered abnormal if they were over-developed, lysed, engorged, or ruptured. Percent abnormally developed eggs was expressed as the total count of abnormally developed eggs divided by the total number of eggs scored. Sperm motility videos were recorded at the beginning and end of the experiment for control sperm. Videos were recorded post-thaw, but prior to fertilization, for cryopreserved sperm.

Two independent analyses were performed to analyze successful fertilization. In the final model for percent fertilized, treatment (cryopreserved vs. control sperm) was included. A fixed weight using the nlme package was applied to difference in female. The second analysis, comparing percent abnormally developed eggs, also included treatment as a fixed effect. Female was included as a random effect with no weighted variance structure applied. Sperm concentration and the interaction of treatment and concentration were not included in either analysis.

RESULTS

Cryoprotectants

Sperm motility was significantly affected by cryoprotectant type and concentration, and there was no interaction of cryoprotectant and concentration in the best fit model (Table 3, Figure 3). Overall, an increase in cryoprotectant concentration caused a significant decrease in sperm motility. DMSO and PG yielded significantly greater motility compared to GLY. Therefore, the following experiment on exposure time of different cryoprotectants included DMSO and PG at 5% and 10% to confirm the type and concentration of cryoprotectant that resulted in the highest motility and to determine whether exposure time to cryoprotectant was important.

Exposure time to cryoprotectants did not have a significant effect on sperm motility (Figure 4). As in the previous experiment, type and concentration of cryoprotectants significantly affected sperm motility (Table 3, Figure 4). The same pattern regarding percent motility of DMSO > PG, and 5%>10%, persisted over each exposure time, with no significant interaction. In general, there was a decrease in percent motility with increased exposure time, therefore a standard of DMSO at 5% with the lowest exposure time, 10-13 minutes, was used for all subsequent experiments (Figure 4).

Freezing and Thawing

Colder freezing endpoint temperatures yielded significantly higher percent motility (Table 3, Figure 5B). Freezing rate and thawing temperature had no significant effect on sperm motility (Figure 5A & 5C). No significant interactions were found between freezing rate, endpoint temperature, or thawing temperature. In general, a slower freezing rate and higher thawing temperature yielded higher percent motility (Figure 5A & 5C). Therefore, a freezing rate of -3 ℃ min^{-1} to an endpoint temperature of -60 ℃ before being plunged into liquid nitrogen (-196 ℃) for storage, followed by thawing at 40 ℃ was selected as the standard freezing protocol for the following fertilization experiment.

Table 3. *Final model using GLMM with associated coefficients* |The table below shows the final model used per experiment. Statistical analysis was performed using R statistical analysis program nlme. Corrected Akaike information criterion (AICc) was used to select the most parsimonious model. (MOT) Percent motile sperm, (PF) Percent of fertilized eggs, and (PA) Percent of abnormally developed eggs.

Experiment & final model	Coefficients	Estimate ± SE	t-value	P-value
Cryoprotectants:				
Cryoprotectants and concentrations				
MOT ~ cryoprotectant + concentration + weighted (1|male)				
	Intercept	0.87 ± 0.06	15.09	<0.001
	Cryoprotectant (Reference: dimethyl sulfoxide)			
	propylene glycol	-0.11 ± 0.06	-1.68	0.10
	glycerol	-0.39 ± 0.06	-6.17	<0.001
	Concentration	-0.03 ± 0.004	-8.80	<0.001
	Degrees of freedom: 56			
Cryoprotectants and exposure time				
MOT ~ cryoprotectant + concentration + weighted (1|male)				
	Intercept	0.72 ± 0.07	10.93	<0.001
	Cryoprotectant (Reference: dimethyl sulfoxide)			
	propylene glycol	-0.16 ± 0.04	-3.93	<0.001
	Concentration	-0.04 ± 0.006	-6.85	<0.001
	Degrees of freedom: 37			
Freezing and Thawing:				
Freezing rate, endpoint temperature, and thawing temperature				
MOT ~ endpoint temp + 1 | male				
	Intercept	0.05 ± 0.01	6.10	<0.001
	Endpoint temperature	-0.01 ± 0.003	-2.96	0.004
	Degrees of freedom: 158			

Table 3. *Final model using GLMM with associated coefficients* |continued

Experiment & final model	Coefficients	Estimate ± SE	t-value	p-value
Fertilization:				
Percent fertilized				
PF ~ treatment + weighted (1| female)				
	Intercept	0.63 ± 0.03	24.42	<0.001
	Treatment (Reference: control sperm)			
	Cryopreserved Sperm	-0.61 ± 0.03	-20.35	<0.001
	Degrees of freedom: 33			
Percent abnormally developed				
PA ~ treatment + 1| female				
	Intercept	0.12 ± 0.03	4.26	<0.001
	Treatment (Reference: control sperm)			
	Cryopreserved Sperm	-0.08 ± 0.02	-3.76	<0.001
	Degrees of freedom: 31			

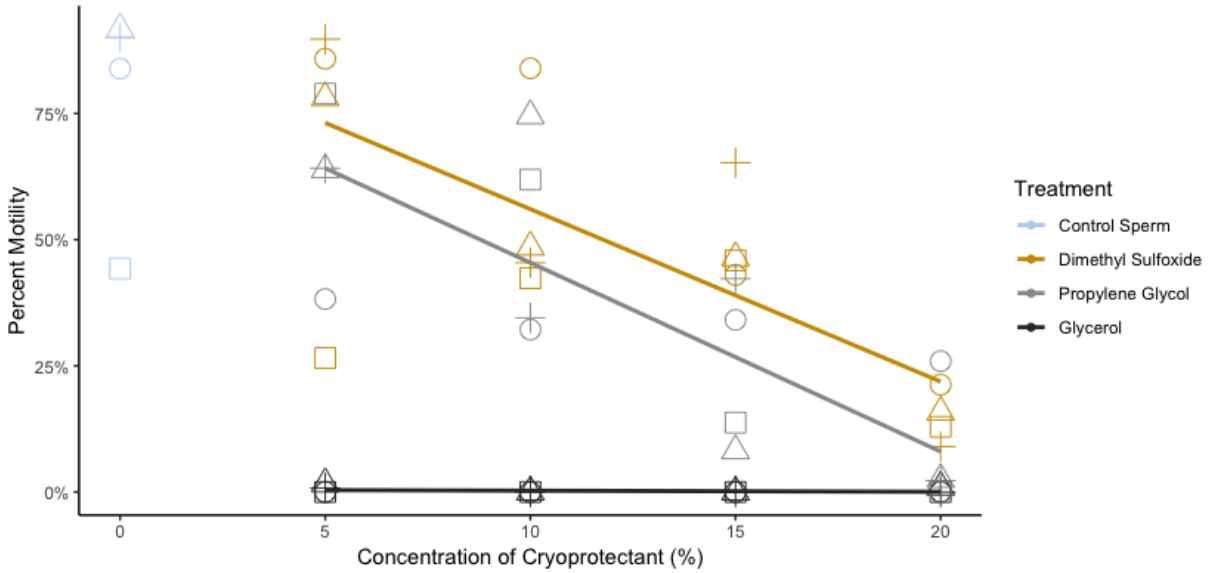

Figure 3. *Effect of cryoprotectant concentrations on percent motility of sperm* (N=4 males). Sperm motility was significantly affected by cryoprotectant type (P <0.001) and concentration (P <0.001). Higher concentrations of cryoprotectants significantly decreased sperm motility (P<0.001). Glycerol had a significant decrease on percent motility (P<0.001). Males are represented by different shapes. One control male (square) had <50% motility, it is unclear if this was due a poor sample video or high variation in male sperm. Male was included as a random factor for the final analysis.

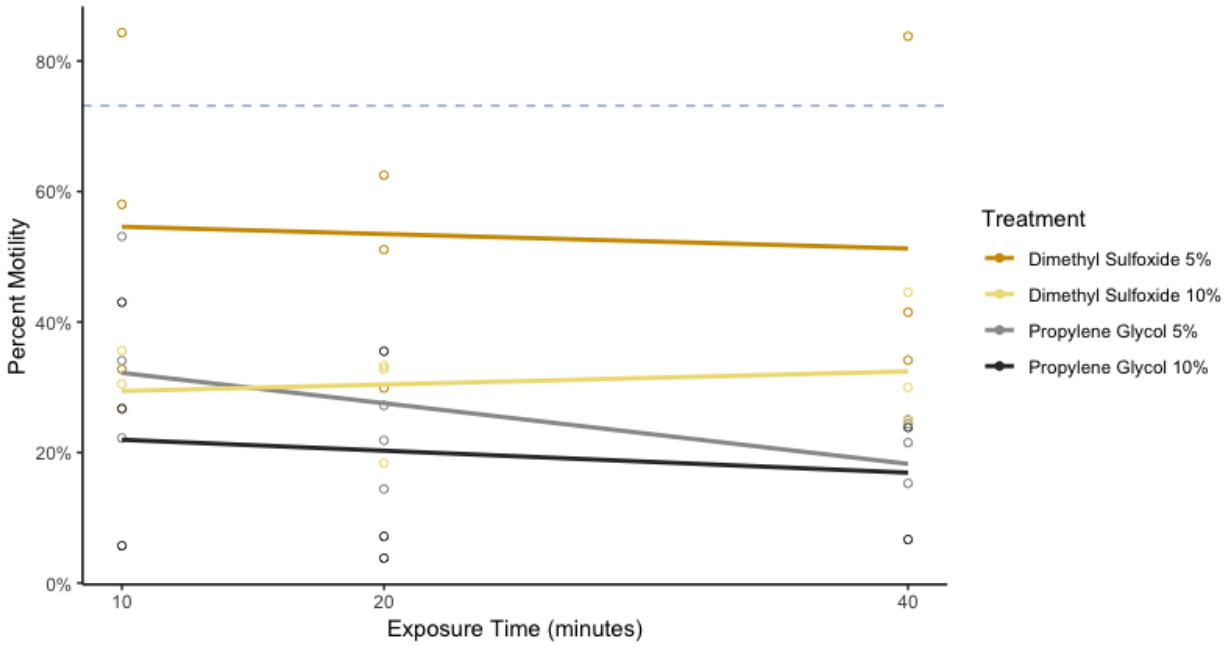

Figure 4. *Effect of exposure time on percent motility* (N= 3 males). Type and concentration of cryoprotectants significantly affected sperm motility (Type: P <0.001; Concentration: P <0.001). No significant difference in motility was observed with an increase in exposure time (P = 0.054). Dashed line represents mean motility of sperm, 72.9% ± 19%, without the addition of cryoprotectants.

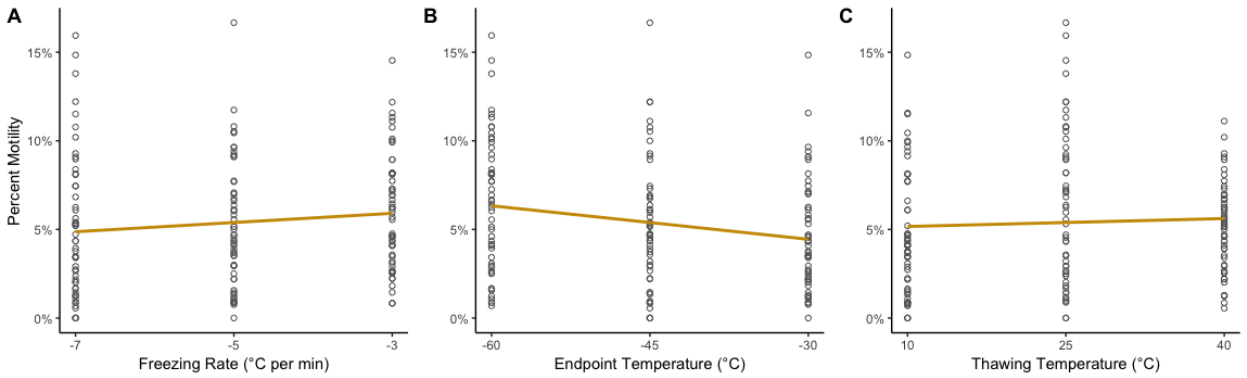

Figure 5. *Effects of A) freezing rate, B) endpoint temperature, and C) thawing temperature on sperm motility* (N=3 males). A) no significant relationship was found between freezing rate, however higher mean motility is seen in slower freezing rates (*P*=0.11), B) Colder endpoint temperatures had significantly higher percent motility (*P*= 0.004), and C) no significant pattern was found in thawing temperature, however higher mean motility is seen using higher thaw temperatures (*P* = 0.48).

Fertilization

The percentage of eggs fertilized by cryopreserved sperm was significantly lower than the percentage of eggs fertilized by control sperm (Table 3, Figure 6A). Sperm concentration and the interaction of cryopreserved sperm and concentration had no significant effect on the percent of eggs fertilized. In general, an increase in sperm concentration increased the percent of eggs fertilized (Figure 6A). Overall, the highest average percent of eggs fertilized using cryopreserved sperm was 3% ± 4% SD with a post-thaw motility of 5% ± 4.3% SD using a sperm concentration of 1×10^6 sperm mL^{-1}. The highest percentage of fertilized eggs using cryopreserved sperm was 12.2% using sperm with a post-thaw motility of 14.2% at a concentration of 1×10^6 sperm mL^{-1}.

There was no significant effect of cryopreservation on the percentage of abnormally developed eggs during fertilization (Table 3, Figure 6B). Similar to percent fertilized, no significant effect of percent abnormally developed eggs was seen with an increase in sperm concentration (Figure 6B). However, in general, there was an increase in percent abnormally developed eggs with increasing sperm concentrations. In general, sperm motility was lower for cryopreserved sperm than control sperm (Figure 6C).

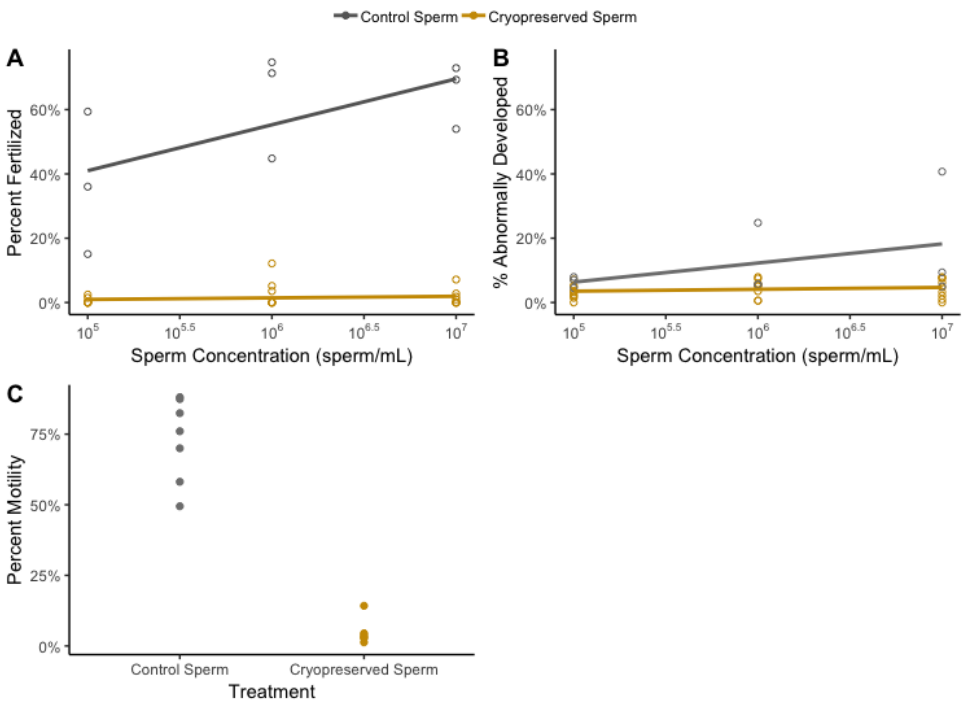

Figure 6. *Percent of eggs fertilized and abnormally developed using cryopreserved vs control sperm at different concentrations* (N=12 male-female crosses). A) Cryopreserved sperm significantly decreased the percent of eggs fertilized compared to control sperm (P <0.001) B) Cryopreserved sperm had significantly lower percent of abnormally developed eggs than control sperm (P <0.001). C) Percent motility was lower for cryopreserved sperm compared to control sperm. This lower sperm activity is likely why percent fertilized and abnormally developed eggs is less when using cryopreserved sperm.

DISCUSSION

During this study, I developed specific tracking parameters to measure pinto abalone sperm motility using a computer assisted program, CASA. Updated with pinto abalone specific parameters, CASA provides the pinto abalone restoration effort with a means to track sperm motility in a reliable, efficient, and reproducible manner for future experiments. Within the context of this study, I was able to use CASA to evaluate the effectiveness of three leading cryoprotectants and a range of freezing and thawing combinations to aid in the development of a standard method of cryopreservation for pinto abalone sperm.

Determining a cryoprotectant's threshold between toxicity and effectiveness during the freezing and thawing process is imperative to setting a standard method. Cryoprotectants, while useful, can be toxic if administered incorrectly (Yang et al. 2013). Toxicity of cryoprotectants can manifest in two distinct ways: osmotic and chemical (Fuller, 2004; Pegg, 2002). Osmotic stress occurs when ice crystals begin to form, thereby increasing the solute concentration outside the cell. The rapid water movement out of the cell to lessen the concentration gradient can, in extreme cases, cause lysis of the cell. To avoid osmotic toxicity, it is important to optimize the solute concentration gradient to avoid cell damage. However, the higher the concentration of cryoprotectant used, the increased likelihood of osmotic stress and subsequently chemical toxicity. Chemical toxicity, for any cryoprotectant, will occur at some concentration, however certain cryoprotectants seem to have a higher threshold when paired with certain species (Fuller, 2004; Pegg, 2002).

Cryoprotectant type, concentration, and exposure time must be carefully selected to avoid rendering gametes useless by acute toxicity and must be selected precisely for each species. DMSO, GLY, and PG are commonly used cryoprotectants and have all had different success in other abalone species and in other marine invertebrates such as oysters, clams, barnacles, squid, urchin, corals, and sea cucumbers (Paredes, 2015). Glycerol, while effective with sperm of some abalone species such as *Haliotis*

rufescens (Salinas-Flores et al. 2005), *Haliotis discus hannai* (Kang et al. 2004) and *Haliotis gigantea* (Matsunaga et al. 1983), was toxic to pinto abalone sperm. GLY has also shown toxic effects on farmed *Haliotis laevigata* at concentrations as low as 4%, and *Haliotis diversicolor*, which experienced a 25% drop in motility at 5% (Liu et al. 2014c). *H. kamtschatkana* appears to share this sensitivity to GLY with a dramatic 76% average decrease in motility in response to the addition of GLY at 5%. In my study, DMSO at 5%, regardless of exposure time, had the least detrimental effects on sperm motility for pinto abalone. PG also had less detrimental effects to sperm motility than GLY but yielded slightly lower motility compared to DMSO.

Most published works on marine invertebrate sperm cryopreservation indicate that DMSO is the most successful cryoprotectant (Paredes, 2015). DMSO's ability to scavenge free radical oxygen during the freezing process, and its low temperature sensitivity allowing for high permeability into the cell, have both been suggested as a source of its effectiveness compared to other cryoprotectants (Fuller, 2004). In regards to abalone, DMSO has been used to cryopreserve sperm in other species such as: *H. rufescens* (DMSO 10%; Salinas-Flores et al. 2005), wild and farmed *H. laevigata* (DMSO 6%; Liu et al. 2014c; Zhu et al. 2014)), farmed *Haliotis rubra* (DMSO 6%; Liu et al. 2015a), *H. diversicolor* (DMSO 6% and 8%; Gwo et al. 2002; Tsai & Chao, 1994), and *H. discus* (DMSO 8%; Matsunaga et al. 1983). I used a slightly lower concentration of DMSO for my freezing and thawing experiments compared to these other studies to lessen any toxic effects. However, the most dramatic decrease in percent motility in my study occurred during the freezing and thawing process. During the freezing and thawing experiment, the addition of DMSO on average decreased untreated sperm motility by 18% ± 2% SE, with an additional 52.71% ± 6.2% SE decrease in motility post-thaw. This reduction in post-thaw motility indicates more protection is needed during the freezing and thawing stage of cryopreservation.

Testing higher concentrations of DMSO during the freezing process may prove useful by increasing the number of solutes to protect the cell from damaging ice formation during freezing. And

while my study did see a significant decrease in motility with increasing concentrations of cryoprotectants, this could be alleviated by the addition of sugars as co-cryoprotectants. In marine molluscs, co-cryoprotectants have not yet been systematically evaluated, however in other species such as finfish and livestock the practice is more common (Liu et al. 2015a). The most commonly used co-cryoprotectants alongside DMSO are sucrose and trehalose (Paredes, 2015). It is thought that the addition of co-cryoprotectants provide two benefits: 1) the added sugars form a layer around the cell, lessening the permeability and therefore the osmotic and chemical toxicity of cryoprotectants, and 2) they stabilize the sperm membrane by lessening the occurrence of oxidative degradation of lipids in the membrane (Khalili et al. 2010; Liu et al. 2015a). Based on my results, it appears that the most effective concentration of DMSO is between 5-10 % and I recommend that the use of co-cryoprotectants be tested to lessen acute toxicity effects associated with using an increased DMSO concentration during the freezing and thawing process.

Alternative options of freezing should be considered if the acute toxicity associated with using higher concentrations of DMSO does not outweigh its cryoprotective abilities. With the increasing interest in cryopreservation in the last decade, methods for freezing cells continue to be developed, with little overlap in successful methods for similar species (Gwo, 2000; Liu et al. 2015a; Paredes, 2015). Freezing by liquid nitrogen vapors is increasing in popularity due to the low-cost and high sample throughput, however vapors do not allow for the standardized temperature drops that programmable freezers provide. I chose to use a programmable freezer to allow for controlled temperature drops and, in theory, decreased variability from sample to sample during freezing. Since my study was the first foray into cryopreserving pinto abalone sperm, it was important to establish upper and lower working limits using standardized methods. Yet, *Liu et al.* (2014b) found a lower concentration of cryoprotectant was needed when using liquid nitrogen vapors versus using a programmable freezer with *H. laevigata*, although the reason for this difference was not clear. So, while freezing by liquid nitrogen vapors does not

provide standardized temperature drops, it could provide an alternative to the programmable freezer by allowing the lower cryoprotectant concentration, DMSO at 5%, to be used and potentially lessen sample to sample variation.

Using a programmable freezer, the highest post-thaw mean motility for *H. kamtschatkana* occurred using -3 ℃ min^{-1} in my study, a slower rate than used for *Haliotis iris* (-5 ℃ min^{-1}; Adams et al. 2004), *H. rufescens* (-16 ℃ min^{-1}; Salinas-Flores et al. 2005), and *H. diversicolor* (-12-15 ℃ min^{-1}; Gwo et al. 2002). During cryopreservation, phase change of membrane lipids from liquid to solid creates tension within the membrane known as cold shock. Using a slower freezing rate can lessen the damage associated with cold shock by easing the cell through this phase change. However, freezing rate did not significantly affect pinto abalone sperm motility in my study.

More important to pinto abalone sperm protection during the freezing process was endpoint temperature and the colder endpoint temperatures, -45 or -60 ℃, significantly improved sperm motility. Based on this, I selected an endpoint temperature of -60℃ to use for the final cryopreservation method tested during the fertilization experiment. This endpoint temperature was within the range of other abalone species such as: wild *H. laevigata* (-60 ℃; Espinoza et al. 2010; Ieropoli et al. 2004; Vitiello et al. 2011) and *H. discus hannai* (-80 ℃; Kang et al. 2004). It is possible that the colder endpoint temperature of -60 ℃ prior to placing the samples in liquid nitrogen at -196 ℃ could have reduced rapid formation of intracellular ice crystals. In a review of oyster cryopreservation, most studies saw extensive injury from intracellular ice crystals formation occurring from 0℃ to -40℃. It was suggested that damage can be lessened by a slower freezing rate to avoid cold shock, along with a colder endpoint temperature past -40℃ (Hassan et al., 2014). While, I did not observe a significant interaction between freezing rate and endpoint temperature, my post-thaw motilities were also lower than other studies on abalone that ranged from 35-65% in a recent review by *Liu et al.* (2015a). By increasing the concentration of cryoprotectant, or adding a co-cryoprotectant during the freezing process, I may see increases in percent

motility that could shed new light on my freezing rate and endpoint temperature selection. My study has provided a working baseline of freezing rates and endpoint temperatures that can successfully cryopreserve sperm, however further optimization during the freezing, and subsequently thawing stage, needs to occur.

Currently, there is a growing evidence that thawing temperature is of equal importance to the freezing techniques used, however it is unclear why different thaw temperatures are better suited for certain species over others (Liu et al. 2015a). What is clear is that determining an optimal thawing temperature lessens the chance of ice recrystallization and reduces osmotic stress on sperm (Immerman & Goetz, 2014). Most abalone cryopreservation studies found that higher thawing temperatures (> 40℃) allowed for higher post-thaw sperm quality. This can be seen in *H. diversicolor* (70 ℃; Gwo et al. 2002), wild and farmed *H. laevigata* (50 ℃ & 60 ℃; Liu et al. 2014c; Zhu et al. 2014), *H. rubra* (60 ℃; Liu et al. 2015d), and *H. rufescens* (45 ℃; Salinas-Flores et al. 2005). However, during preliminary experiments using thawing temperatures higher than 40 ℃, I found minimal post-thaw motility for sperm from filial generations of pinto abalone. Since the pinto abalone's response to a high thawing temperature was different than other abalone species, I focused on establishing a working range of temperatures lower than 40 ℃ for experiments with broodstock. Higher thawing temperatures of 25 ℃ or 40 ℃ provided higher post-thaw motility results than 10 ℃, but I recommend that additional experiments be conducted to select a more focused thaw temperature specific for pinto abalone.

To estimate the efficacy at each step of cryopreservation method development, I used sperm motility as the primary metric. While sperm motility is commonly used as indicator of sperm viability, it does not capture potential damage to the cell membrane that can cause unsuccessful fertilization. Other costlier methods, such as flow cytometry, could be used to assess membrane integrity of sperm heads, however this does not detect midpiece and tail damage. Mitochondria held in the midpiece of the sperm provide energy for flagellar action and any undetected damage to the midpiece could contribute to low

fertilization rates (Salinas-Flores et al. 2005). Thus, the true bell weather of sperm viability remains fertilization.

Even with optimized cryopreservation methods, a high proportion of sperm will have damage to essential anatomy such as the acrosome, midpiece, or injury to the membrane. Too account for high likelihood of damaged cells, cryopreservation literature supports using a fertilization curve to determine an effective density of cryopreserved sperm to egg ratio. In my study, at each sperm concentration the percent of eggs fertilized with cryopreserved sperm was lower than with untreated sperm, likely due to the greater proportion of inactive sperm versus active sperm in cryopreserved sperm. It is not uncommon for fertilization with cryopreserved sperm to be less than untreated sperm, however for restoration organizations to use this technique reliably there needs to be higher fertilization than my average percent of eggs fertilized (3% ± 4%), and with more overall consistency, to be beneficial. In a review of marine invertebrate cryopreservation, other abalone studies have seen fertilization success ranging from 48% up to an impressive 94% (Liu et al. 2015a). *Liu et al.* (2014c) even found 84% fertilization with only 38% sperm motility. This indicates that with optimization to my existing methods we will likely see an increase in fertilization.

The hatchery program is first and foremost a restoration effort, used to produce pinto abalone to restock wild populations in Washington. The overall goal is to reach the minimum density needed in the wild for successful reproduction to occur, but not at the cost of damaging the existing population. Cryopreservation is a tool that comes with many benefits to a small-scale restoration program, however this tool should be extensively evaluated at both the phenotypic and genotypic level before being applied to animals that will be released into the wild. Through this study, I have outlined the first attempt into cryopreservation methods for pinto abalone sperm and provided a foundation for further refinement of this method. I have shown that cryopreserved pinto abalone sperm can successfully fertilized eggs, however optimization is still needed to use cryopreservation as a restoration tool.

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