Sablefish (*Anoplopoma fimbria*) sperm: The physiology of activation and the development of a cryopreservation protocol

Douglas A. Immerman

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Committee:

Steven Roberts

Rick Goetz

Graham Young

J. Adam Luckenbach

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University of Washington

Abstract

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Douglas A. Immerman

Chair of the Supervisory Committee:

Associate Professor Steven Roberts

School of Aquatic and Fishery Sciences

Sablefish (*Anoplopoma fimbria*) is a deep-water marine species in the Pacific Ocean with high commercial value. Limited information is available about male reproductive development and sperm physiology in sablefish or how to optimize fertilization and sperm storage in this species for aquaculture. In the present study the conditions required for sablefish sperm activation were investigated and an optimized regime for cryopreservation of sablefish sperm was developed.

In all experiments, the assessment of sablefish sperm was conducted using a computer assisted sperm analysis (CASA) system that determined the velocity and linearity of motile sperm as well as the percent motility. A modified Cortland's solution

was used as an extender in all activation and cryopreservation tests. Sperm were activated with ionic (NaCl, KCl, $MgSO_4$ and $CaCl_2$) and non-ionic (urea and glucose) solutions of varying osmolalities. KCl and NaCl activated sperm at the lowest osmolalities, followed by $MgSO_4$, urea, glucose, and $CaCl_2$. Compared to NaCl and KCl, non-ionic solutions required higher osmolalities for activation. The results indicate that the primary stimulus for sablefish sperm activation is increased osmolality and not the presence/absence of a specific ion.

The effects of several cryoprotectants including dimethyl sulfoxide, propylene glycol and glycerol at two concentrations (5% and 10%) and three freezing rates (-2.5, -5 and -7.5 °C/min) were tested on the cryopreservation of sperm using a Crysalys cryogenic programmable freezing system. There were differences observed in the motility maintained following freezing between all cryoprotectants, but the highest motility after freezing was observed with 10% dimethyl sulfoxide at all freezing rates.

TABLE OF CONTENTS

Page

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LIST OF FIGURES

Figure Number

LIST OF TABLES

Table Number

CHAPTER 1:

General Introduction

1.1. Sablefish

Sablefish (*Anoplopoma fimbria*) is a deep-water species in the Pacific that commands a high commercial value. Sablefish are distributed in the north Pacific Ocean from the Bering Sea South to Baja California and West to Japan at depths ranging from 0-2740 m (Allen & Smith 1988). Eggs and yolk-sac larvae initially reside below 200 m, while later the larvae are found closer to the surface (Mason et al. 1983; Kendall Jr & Matarese 1987) where they stay into the early juvenile life stages (Moser et al. 1994). After one to two years, older fish move to deeper water where they remain as adults. The sablefish spawning season starts in January with a peak in February and can, in some locations, continue into May (Mason et al. 1983).

Life history and egg incubation studies for fisheries management have provided information related to egg development and reproduction in wild-caught female sablefish (Alderdice et al. 1988a; Alderdice et al. 1988b; Kendall Jr & Matarese 1987; Mason et al. 1983). However, not much is known about male reproductive development and sperm physiology or how to optimize sperm use or storage. Currently, there are a few hatcheries in the early phases of sablefish production that need technologies to increase output and efficiency since limitations exist in regard to sablefish sperm. Commercial hatcheries utilize sablefish collected from the wild in which sex ratios can be strongly biased toward females, (Mason et al. 1983) but this may depend on the collection method. The low number of males limits genetic diversity when creating genetically

defined, captive broodstocks, as well as simply having sufficient amounts of milt for fertilization.

1.2. The Need for More Efficient Aquaculture Production

Aquaculture accounts for approximately 50% of the world's seafood consumption and has grown at a rate of 8.8% per year since 1950 making it the fastest growing food-producing sector (FAO 2006). In the United States, seafood consumption has risen over the last century from 11.2 to 16.3 pounds consumed per capita per year (NOAA 2008). In 2004 the United States produced only 1.2% of the world's seafood through aquaculture (FAO 2006). Due to low production and high consumption, the United States is a large importer of seafood. Relying on foreign producers introduces issues, such as reduced regulatory standards, thereby inviting increased antibiotic and pesticide contamination of food products (Doyle & Erickson 2008). Increased domestic production could help provide a safe food supply by ensuring that stricter regulatory standards are met on all seafood products. To meet this goal, aquaculture operations need to run at maximum efficiency. Aquaculture has the inherent disadvantage of low profit margins. Because of this, improvements in efficiency must be made to increase profits to their maximum capacity. For some fish species, many aspects of production have already been investigated to improve the efficiency of aquaculture operations, such as food formulations to improve conversion ratios, water quality to promote health, and water temperature to improve growth (Trushenski et al. 2013; Terjesen et al. 2013; Hernandez et al. 2007). However, for many species, numerous aspects of reproduction and fingerling propagation are still poorly understood, including egg and sperm quality.

1.3. Sperm Management

Sperm can be a limiting factor in aquaculture in a variety of ways depending on the species. In some instances, facilities require enhanced genetic diversity. This is especially true for research and in the creation of captive broodstocks (i.e., spawning stocks). In these situations, obtaining sperm from as many males as possible is optimal. Though many males may be "running" (i.e., producing and releasing sperm) at the time of reproduction, there may not be high enough volumes of milt produced by each male. Sperm concentrations in fish milt are often very high, and when diluted and distributed evenly throughout the eggs, small volumes of sperm can be used to fertilize large volumes of eggs. However, this requires a thorough understanding of the conditions under with sperm can be diluted as well as the requirements for sperm activation.

A common technique used in aquaculture is "out-of-season" spawning, also referred to as "cycled out of season." This is where environmental cues are manipulated to induce the fish to spawn at different times of the year. With this strategy, having cryopreserved (cryogenically frozen) sperm from a variety of males of genetically diverse backgrounds can allow for more females to be cycled without having to cycle males as well. In addition, cryopreservation allows use of the same sperm to fertilize eggs stripped from different females with different spawning schedules months or even years apart. Where situations arise, such as those mentioned above, 'sperm management' is required to fulfill the production potential. Sperm management can encompass a variety of techniques including storage, extension, and quality assessment (Rurangwa et al. 2004). Some examples of these technologies are extenders for dilution and short-term storage,

cryopreservation for long-term storage, and the use of computer-assisted sperm analysis (CASA) for evaluating sperm quality (Billard et al. 2004; Kime et al. 2001).

A better understanding of sperm management practices for sablefish will allow for greater use of a limited sperm supply, increased genetic variation of broodstocks, increased success in sperm transport, reduction in the number of broodstock that must be maintained especially in photoperiod manipulated facilities, and synchronization of gamete availability (Riley et al. 2004; Suquet et al. 2000).

1.4. Anatomy of the Male Reproductive Tract

In fish, the testis is made up of two lobes and consists of an outer wall, and tubular and intertubular compartments (Koulish et al. 2002; Billard 1986). The intertubular compartment is comprised of neural and connective tissues, blood and lymphatic vessels, macrophages, mast cells and steroidogenic Leydig cells (Schulz et al. 2010). At different stages of development the tubular compartment, containing the germinal epithelium, may have somatic Sertoli cells and germ cells. The spermatogenic capacity is dependent on the number of Sertoli cells present (Matta et al. 2002). The Sertoli cells are vital to the regulation of spermatogenesis as they are in very close proximity to the germ cells and offer structural and metabolic support (Matta et al. 2002; Schulz et al. 2010). Germ cells give rise to the spermatogonia through spermatogenesis in cysts created by the Sertoli cells within the lobules of the testes (Billard 1986; Nobrega et al. 2009).

The sperm duct is comprised of the genital papilla and the posterior duct of both lobules. This area is void of germ and Sertoli cells and is where spermatozoa collect for some species (Billard 1986).

1.5. Spermatozoan Morphology

Spermatozoan morphology can vary between species. This variability can be explained by the adaption of species to specific habitats (Billard 1986). Spermatozoa of teleost fish all have a spherical nucleus (head), condensed chromatin, nuclear fossa, a midpiece and a flagellum (Gwo et al. 2006; Gwo et al. 2004; Gwo et al. 2005; Ohta et al. 1994). An acrosome (cap) is often present on the head of vertebrate sperm. However, an acrosome is often absent in marine fishes (Ohta et al. 1994). The mitochondria are located at the base of the sperm head (Gwo et al. 2005). Sperm of teleost fish that practice external fertilization undergo rapid osmotic shock and must maintain high energy stores that allow rapid motility for short periods of time and, as a result, many fish with external fertilization exhibit group spawning behavior to optimize the potential fertilization success (Billard 1986).

1.6. Spermatogenesis

Spermatogenesis is a process where diploid primordial germ cells called spermatogonia, develop through mitosis and meiosis into large numbers of highly differentiated mature, haploid spermatozoa (Billard 1986; Nobrega et al. 2009). Spermatogenesis is made up of three different phases. The initial phase of spermatogenesis can be considered the spermatogonial (mitotic) phase where diploid

spermatogonia divide mitotically. The mitotic phase encompasses different generations of spermatogonia including type-A spermatogonia, intermediate spermatogonia and type-B spermatogonia. The subsequent meiotic phase, spermatogenesis, is where type-B spermatogonia are reduced to haploid cells through meiotic division. The first division gives rise to primary spermatocytes and the second division yields secondary spermatocytes (Schulz et al. 2010). The last cell division results in the creation of spermatids. In the spermiogenic phase, the motile spermatozoa mature from the haploid spermatids (Nóbrega et al. 2009).

1.7. Hormonal Regulation of Spermatogenesis

The regulation of testicular development can be largely attributed to the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These two hormones are produced in the pituitary. FSH is the regulator of Sertoli cell function, whereas LH is responsible for the regulation of steroidogenesis by the Leydig cell (Schulz et al. 2010). In salmonids, levels of LH begin to increase with germ cell meiosis. These levels increase substantially close to the spawning season. By the spawning season, levels of FSH have already decreased but are high during periods of spermatogonial proliferation and spermiation (Campbell et al. 2003).

The steroid hormones, estrogens, androgens and progestins are produced at specific times in the testes depending on the age and the stage of gonadal maturation. An increase in LH stimulates the conversion of cholesterol to pregnenolone within the mitochondria and pregnenolone serves as a substrate for the other reproductive steroids (Young, G. et al. 2005). Androgens, such as testosterone and 11-ketotestosterone, are

expressed in the testes and increase during spermatogenesis but subsequently decrease during spermiation (Schulz et al. 2010). These androgens promote spermatogonial proliferation, spermatocyte formation and, in conjunction with progestins, are involved in puberty initiation and spermiation (Remacle 1976; Fostier et al. 1983; Borg 1994; Miura et al. 1991; Cavaco et al. 2001; Ueda et al. 1985). Progestins, such as 17α,20β,21-trihydroxy-4-pregnen-3-one (DHP), are important in male reproduction as they induce spermiation and increases milt production (Schulz et al. 2010). Although present at low levels, 17β-estradiol is found in the serum of male teleosts and increases at the beginning of the reproductive cycle of trout (Schulz et al. 2010). Estrogen receptors are present in haploid male germ cells as well as in the Sertoli and Leydig cells (Menuet et al. 2002; Wu et al. 2001; Bouma & Nagler 2001; Chang et al. 1999) suggesting that estrogen plays a role in the regulation of testicular gene expression. All three types of steroids have been shown to induce spermatogonial proliferation (Young, G. et al. 2005)

Steroids are carried through the blood by sex hormone binding proteins (SBPs). Not only do SBPs carry the sex hormones, they also prevent them from being excreted (Devlin & Nagahama 2002; Young, G. et al. 2005).

1.8. Sperm Activation

In situ, sperm of freshwater and marine fish is quiescent in the seminal fluid (~300 mOsm/kg) and in solutions isotonic to the seminal fluid (Morisawa & Suzuki 1980). The sperm density in the seminal fluid is high which assists in maintaining acute O_2 depletion and contributes to immotility (Cosson et al. 2008). At spawning, however, the sperm are released into widely varying environments depending on whether they are

marine or freshwater species. Because of this, species have developed very different mechanisms for optimal activation and performance suited to the environments in which they will be released and suited to the reproductive behavior being exhibited by that species. In seawater acclimated tilapia (*Oreochromis mossambicus*) sperm activation requires $CaCl_2$, while species from the family Cyprinidae exhibit motility in response to low osmotic pressure (Linhart et al. 1999; Alavi & Cosson 2006). Further, in some species K^+ acts as an activation inhibitor. However, this is most often noted in species from Salmonidae and Acipenceridae (Cosson 2004).

Activation of marine fish sperm varies between species but is generally dependent on an external osmotic rise. For example, the optimal range of osmolality for the activation of halibut (*Hippoglossus stenolepis*) sperm is 900-1100 mOsm L^{-1} (Billard et al. 1995), while turbot (*Scophthalmus maximus)* sperm can be activated in hypertonic sugar solutions with an optimal osmotic pressure of 400-900 mOsm (Chauvaud et al. 1995). Once underway, activation can also be halted by changing to an isotonic environment, and can be initiated again by exposure to a hypertonic solution (Takai & Morisawa 1995). In marine fish sperm that can be activated by hypertonicity alone, an increase in osmolality causes an osmotic exit of water from the cell through local aquaporins, causing membrane stretching (Cosson et al. 2008). The stretching of the membrane triggers a response by stretch-activated channels, thus increasing local permeability allowing for increased ion conductivity through channels and for water to move rapidly through porins (Cosson et al. 2008; Yang & Sachs 1993). Activated aquaporins are capable of increasing water permeability up to three fold. The increase in the internal ionic concentration initiates an auto-catalytic effect along the flagellar

membrane, thus self-amplifying the ionic concentration effect. The increased internal ionic concentration that occurs through the rapid pumping of water out of the cell and transport of ions into the cell, causes ionic concentrations to reach optimal levels for the dynein to act as a motor, thus initiating flagellar beating (Cosson et al. 2008).

Peptides released from eggs to induce sperm chemotaxis have been well studied in a number of marine invertebrates (Kaupp et al. 2006; Ward & Kopf 1993). This response has also been noted in aquatic vertebrates such as Atlantic salmon (*Salmo salar)*, brown trout (*Salmo trutta)* and Pacific herring (*Clupea pallasii)* (Yeates et al. 2013; Morisawa 2008). Chemotaxis decreases the linearity of the sperm's swimming path by decreasing flagellar symmetry. These peptides stimulate motility and the metabolism of phospholipids, while increasing oxygen consumption, cAMP, cGMP, Ca^{2+}, and pH (Ward & Kopf 1993). Ca^{2+} changes flagellar beat symmetry by acting on the axoneme (Brokaw 1991). The binding of oligopeptides to the receptor guanylyl cyclase initates a cascade of events that lead to a hyperpolarization of the cellular membrane and the opening of calcium channels (Kaupp et al. 2008). This signaling uses the Ca^{2+} that is prevalent in seawater.

1.9. Cryopreservation

Depending on the species, unactivated sperm can be stored from hours to days without freezing. However, proper cryopreservation can extend the length of sperm storage. In humans, successful fertilizations and subsequent live births using sperm cryopreserved for over 28 years have been documented (Feldschuh et al. 2005). Past studies on marine fish sperm have examined the effects of cryoprotectants, diluents and

freezing ramp rate along with freezing temperature (Billard et al. 2004; Butts et al. 2010; Suquet et al. 2001). Cryopreservation of sperm is typically achieved through the immersion into liquid nitrogen of specialized chambers that hold straws or vials. These chambers can control, through a computer interface, the rate at which the sperm is frozen. To establish a suitable cryopreservation protocol, there are three main components that are typically adjusted to suit the different physiology of sperm from different species of fish: diluent, cryoprotectant, and the rate at which the sperm is frozen.

As the name implies, the cryoprotectant protects the sperm from being damaged by the freezing process. Cryoprotectants such as glycerol and dimethyl sulfoxide (DMSO) penetrate the cellular membrane to improve protection (Pegg 1976) and have been used in the cryopreservation of fish sperm. According to Nash (1962), suitable cryoprotectants must possess three important attributes: low toxicity, penetrative power and the ability to resist precipitating out below freezing temperatures. For teleost sperm, the most successful cryoprotectants are DMSO (5%-20%), glycerol (10%-20%) and propylene glycol (10%) (Cabrita et al. 2010). Without a protectant there is very little likelihood of sperm survival due to damage by ice formation (Chao 1991). In addition to protection from ice formation, cryoprotectants also prevent or reduce temperature shock, dehydration and help stabilize protein in aqueous solutions (Chao 1991). However, in the freezing process, cryoinjuries can occur. Disruption to the cristae of the mitochondria, swollen plasma membrane, and coiled axoneme have been observed in Atlantic croaker (Gwo & Arnold 1992). Cryoinjuries to the sperm can significantly reduce the quality of the sperm, thus reducing fertilization rates (Suquet et al. 2001). At higher temperatures, however, cryoprotectants can cause protein denaturation and cellular toxicity (Muchlisin

2005). This makes timing of equilibration with the cryoprotectant very important. Enough time must be allowed for equilibration of the cryoprotectant into the cell but not for cell toxicity to occur.

DMSO, is a very popular cryoprotectant for use in animal cells and has been used successfully at concentrations ranging from 5-15% for the cryopreservation of sperm of a number of fish species including, Arctic char (*Salvelinus alpinus,* Richardson et al. 2000), barramundi (*Lates calcarifer,* Leung 1987), grouper (*Epinephelus tauvina*, Withler & Lim 1982), Atlantic croaker (*Micropogonias undulatus,* Gwo et al. 1991) and yellowtail flounder (*Pleuronectes ferrugineus,* Richardson et al. 1995). However, the success of cryoprotectants may be species-specific since glycerol has offered better protection than DMSO in European catfish (*Silurus glanis*, Linhart et al. 1993), summer flounder (*Paralichthys olivaceus*, Zhang et al. 2003), and yellowfin seabream (*Acanthopagrus latus*, Gwo 1994) sperm. In contrast, glycerol failed to offer any protection to black grouper sperm (*Epinephelus malabaricus*, Gwo 1993). Propylene glycol offered low to marginal protection for summer flounder (Zhang et al. 2003), but successfully protected winter flounder (*Pseudopleuronectes americanus*, Rideout et al. 2003), haddock (*Melanogrammus aeglefinus)* and Atlantic cod (Rideout et al. 2004) sperm.

In addition to a cryoprotectant an appropriate extender (diluent) is also important in the freezing process. Numerous extenders have been used for fish sperm and are generally composed of similar chemicals. Almost all contain NaCl, KCl, $CaCl_2$ and Mg^{2+} (in the form of either $MgCl_2$ or $MgSO_4$), along with a buffer for pH stabilization and often a sugar source such as glucose (Muchlisin 2005; Cabrita et al. 2010). All extenders must be iso-osmotic to the seminal fluid so activation is not initiated (Morisawa 2008).

Optimal freezing rate can be very species dependent. For marine fish species optimal freezing rates vary significantly from 5°C/min for cod (Mounib et al. 1968) and 8°C/min for hirame (Tabata & Mizuta 1997) to 99°C/min for turbot (Dreanno et al. 1997) with many species falling in between.

OBJECTIVES OF STUDY

- Determination of the conditions required for sablefish sperm activation
  - Activation of sperm with ionic solutions
    - NaCl
    - KCl
    - $MgSO_4$
    - $CaCl_2$
  - Activation of sperm with non-ionic solutions
    - Glucose
    - Urea
  - Determine the osmolality required to initiate sperm activation with ionic and nonionic solutions
  - Assess the linearity of sperm activated with ionic and non-ionic solutions

- Develop an optimized regime for cryopreservation of sablefish sperm
  - Determine optimal cryoprotectant
    - Dimethyl sulfoxide
    - Propylene glycol
    - Glycerol
  - Determine optimal cryoprotectant concentration
    - 5%
    - 10%
  - Determine optimal freezing rate
    - 2.5° C/min to -40 °C
    - 5° C/min to -40 °C
    - 7.5° C/min to -40 °C

CHAPTER 2:

The activation and cryopreservation of sablefish (*Anoplopoma fimbria*) sperm

Douglas A. Immerman[a], Frederick W. Goetz[b*]

[a] School of Aquatic & Fishery Sciences, University of Washington, 1122 NE Boat St., Seattle, WA 98195, USA

E-mail: Immerd@uw.edu

[b] National Oceanic and Atmospheric Administration (NOAA), Manchester Research Station, 7305 E. Beach Dr., Manchester, WA 98366 USA

*Corresponding author: Frederick W. Goetz

Phone: 001-360-871-8322

Fax: 001-206-842-8364

E-mail: Rick.Goetz@NOAA.gov

Address: National Oceanic and Atmospheric Administration (NOAA), Manchester Research Station, 7305 E. Beach Dr., Manchester, WA 98366 USA

2.1.0. Introduction

Sablefish (*Anoplopoma fimbria*) is a deep-water marine species in the Pacific Ocean with high commercial value (Sumaila et al. 2007). Life history and egg development studies have provided information related to early larval development and female reproduction (Alderdice et al. 1988a; Alderdice et al. 1988b; Kendall Jr & Matarese 1987; Mason et al. 1983). However, there have been no reports on male reproductive development and sperm physiology in sablefish or how to optimize fertilization or sperm storage in this species.

The high demand and commercial value of sablefish has leveraged the development of aquaculture for this species. However, this is a relatively new endeavor and certain aspects such as captive broodstocks and genetic selection are just being developed. Broodstocks are frequently obtained from the wild and with current fishing methods, sex ratios can be highly skewed toward females (Mason et al. 1983). Thus, sperm is frequently limiting and impacts many of the management practices required in aquaculture including the maintenance of genetic variation in crosses and the availability of sperm for out-of-cycle spawning (Suquet et al. 2000). A better understanding of sperm activation and storage in sablefish would greatly facilitate the culture of this species.

In situ, sperm of freshwater and marine fish is quiescent in the seminal fluid (~300 mOsm/kg) (Morisawa & Suzuki 1980). The sperm density in the seminal fluid is high which assists in maintaining acute O_2 depletion and contributes to immotility prior to activation (Cosson et al. 2008). In freshwater fish the mechanism of sperm activation can vary between species. For salmonids, K^+ plays an important role by inhibiting activation (Cosson 2004). In fish species, the sperm can be kept immotile outside of the

fish if held in a solution that is isotonic to the seminal fluid and can be activated in a solution that is hypertonic to the seminal fluid (Morisawa 2008). Once underway, activation can also be halted by changing to an isotonic environment, and can be initiated again by exposure to a hypertonic solution (Takai & Morisawa 1995).

Sperm cryopreservation has been achieved in many freshwater and marine fish species and requires an extender for diluting the sperm and a cryoprotectant to protect the sperm during freezing (Stein & Bayrle 1978; Suquet et al. 2000). Cryoprotectants such as glycerol and dimethyl sulfoxide (DMSO) penetrate the cellular membrane to improve protection (Pegg 1976). According to Nash (1962), suitable cryoprotectants must possess three important attributes: low toxicity, penetrative power and the ability to resist precipitating out below freezing temperatures. For teleost sperm, the most successful cryoprotectants are DMSO (5%-20%), glycerol (10%-20%) and propylene glycol (10%) (Cabrita et al. 2010). In addition to a cryoprotectant, an appropriate extender (diluent) is also important in the freezing process. Numerous extenders have been used for fish sperm and are generally composed of similar chemicals. Almost all contain NaCl, KCl, $CaCl_2$ and Mg^{2+} (in the form of either $MgCl_2$ or $MgSO_4$), along with a buffer for pH stabilization and often a sugar source such as glucose (Muchlisin 2005; Cabrita et al. 2010). All extenders must be isosmotic to the seminal fluid so activation is not initiated (Morisawa 2008). Successful freezing rates vary significantly from 5°C/min-99°C/min (Suquet et al. 2000; Tiersch et al. 2007).

In the present study we determined the conditions required for sablefish sperm activation, and developed an optimized regime for the cryopreservation of sablefish sperm. The results of this study provide a means to more efficiently use sablefish sperm

for fertilization and also to better manage it for aquaculture operations that require long-term storage.

2.2.0. Methods

2.2.1. Milt Collection

Male sablefish (average weight 2.8 kg) were caught via hook and line 20 km off of Westport, WA (N 047 17 W 124 52) at a depth of approximately 200 m. They were transported to the Manchester Research Station (Port Orchard, WA) and held at 5-6° C in 3 m round tanks supplied with recirculating seawater. During the spawning season (January to April) males were implanted in the right dorsal sinus with Ovaplant (Western Chemical, Ferndale, WA), a salmon gonadotropin releasing hormone analogue, at 50-60 μg/kg body weight. This treatment initiated or enhanced spermiation within one-two weeks. To collect milt, spermiating males were anesthetized with Tricaine-S (Western Chemical Ferndale, WA) and strip-spawned. A 5 ml pipette was used to collect the milt directly from the dried vent and the milt was placed in a Whirlpak bag (Nasco, Modesto, CA) to which pure oxygen was added over the milt prior to closing. Milt was held under these conditions at 4° C until experimentation.

2.2.2. Extender

For sperm dilution, a modified Cortland's solution was selected and used (Table 1) (Goetz & Coffman 2000). The average pH of the sablefish milt was 7.7 ± 0.12 (N=6) as determined using a handheld pH meter (Hanna Ann Arbor, MI). Based on this, the pH

Table 1. Formula for modified Cortland's solution (Goetz & Coffman 2000)

	Molarity	g/L
NaCl	124.1 mM	7.25
KCl	5.1 mM	0.38
$CaCl_2.2H_2O$	1.6 mM	0.235
$MgSO_4.7H_2O$	1 mM	0.246
Glucose	5.6 mM	1
HEPES	20 mM	4.77
NaOH	1 M	7.7 ± 0.12

of the extender was adjusted to 7.7 for all experiments. To reduce the number of possible variables no other extenders were tested.

2.2.3. Sperm Activation and Analyses

Sablefish sperm density was determined to be 2.0 x 10^{10}- 2.5 x 10^{10} ml^{-1} using a hemocytometer. Given the recommended sperm concentration (10^{4}-10^{6} ml^{-1}) for computer assisted sperm analysis (CASA) (Kaupp et al. 2003; Spehr et al. 2004; Riffell et al. 2002), sablefish milt was diluted in the Cortland extender at a ratio of 1:100 and held overnight in 50 ml falcon tubes at 4°C. For sperm analysis, the motility was recorded at 97 frames per second on a microscope-mounted (Olympus IM, Center Valley, PA) video camera (Allied Prosilica GC, Stadtroda, Germany) at 10x magnification with dark field illumination. To keep the sperm from sticking to the glass slide, 0.2 µl of a 10% gelatin solution derived from cold-water fish skin (#G7765 Sigma-Aldrich, St. Louis, MO) was placed into the channel before adding the sperm. To activate the sperm, 100 µl of diluted sperm (1:100) was mixed with 900 µl of activating solution and 1.5 µl of the activated sperm mixture was immediatly loaded into a 20-micron channel of a Leja sperm analysis slide (Leja, Netherlands).

High osmolality stock solutions of KCl, NaCl, urea, glucose, $MgSO_4$, and $CaCl_2$ were made and diluted to create working solutions ranging from 400-1050 mOsm/kg in increments of 25 mOsm/kg. Sperm was activated with the working solutions at each osmolality starting with the lowest osmolality solution for which activation was observed and continuing incrementally through the next three higher concentrations and again at 800 mOsm/kg and 1050 mOsm/kg. Sperm from 2-7 males were tested in triplicate with

each solution. The video files of the sperm were opened in ImageJ (Schneider et al. 2012) and a variance filter was applied to remove background gradation and the threshold was adjusted to observe white sperm against a black background. The video was then analyzed at 16-17 seconds into activation using the CASA plugin (Wilson-Leedy & Ingermann 2007) to determine the percent motility, curvilinear velocity and linearity. Linearity is defined as straight-line velocity over average path velocity and describes the curvature of the sperm path. All velocity units were measured in μm/s.

2.2.4. Cryopreservation

Sperm was diluted in the Cortland extender at a 1:50 ratio and held overnight at 4°C. Cryoprotectant at two times the desired final strength was slowly added to the sperm over a period of 10 min to create a sperm dilution of 1:100. To accomplish this, the sperm was held in a plate on a shaker providing continuous rotary agitation as the cryoprotectant was added. Cryoprotectant was added to the sperm in a coldroom maintained at 4^{o}C. Following the addition of the cryoprotectant, the sperm was loaded into 0.25 ml cryopreservation straws and frozen at different rates. Cryoprotectants tested were glycerol, propylene glycol and DMSO at 5% and 10% concentrations. For all six cryoprotectant treatments, three freezing rates (Table 2) were tested. All freezing protocols started at -4°C and were performed using the Crysalys PTC-9500 (Biogenics Napa, CA) cryogenic programmable freezing system. Straws were loaded into the freezing chamber and the program was initiated. After the freezing was completed, the straws were held in liquid nitrogen for 7 days before assessment. Sperm was thawed by holding the straw in 28°C water for seven seconds (Butts et al. 2010). The sperm (100 μl)

Table 2. Freezing rates tested. LN=liquid nitrogen

Starting temp	Ramp 1	Ramp 2	Final step
-4 °C	2.5 °C/min to -40 °C	5° C/min to -60 °C	Plunge into LN
-4 °C	5 °C/min to -40 °C	5° C/min to -60 °C	Plunge into LN
-4 °C	7.5 °C/min to -40 °C	5° C/min to -60 °C	Plunge into LN

was then activated in 900 µl of 1050 mOsm/kg NaCl and assessed using CASA. For controls, sperm activation was also tested by CASA prior to dilution in the extender, prior to adding the cryoprotectant, and following the addition of the cryoprotectant but prior to freezing. This also allowed us to verify that the cryoprotectant at 5 and 10% concentrations was not toxic to the sperm following 10-13 minutes of exposure.

2.2.5. Statistical Analyses

Statistical analyses were performed using XLSTAT (Version 2013.5.04 Addinsoft, Inc., Brooklyn, NY, USA), a plugin for Microsoft® Excel for Mac 2011 (Version 14.3.8). All data sets were tested for normality with the Shapiro-Wilk test and for homogeneity of variances using Bartlett's test prior to other analyses. To compare the results of the different activating solutions, each data set was fit to a 4-parameter logistic curve to determine the 50% activation point ("C value"). These values were compared by ANOVA. To analyze the cryoprotectant data, post-thaw motility was divided by the motility measured prior to freezing ("motility maintained"), and this percentage was arcsine square root transformed. For all subsequent analyses, an ANOVA was performed followed by a Tukey's post hoc test for treatment comparisons and a Dunnett's post hoc test for comparisons to control values.

2.3.0. Results

2.3.1. Activation

KCl activated the sperm at the lowest osmolality (550-625 mOsm/kg) followed by NaCl (550-625 mOsm/kg), $MgSO_4$ (600-650 mOsm/kg), urea (625-700 mOsm/kg), glucose (625-700 mOsm/kg) and $CaCl_2$ (650-700 mOsm/kg) (Fig. 1). Based on the analysis of the 50% activation value, KCl required the lowest osmolality for activation (avg. 613±29.4 (SD) mOsm/kg), followed by NaCl (avg. 633±31.3 mOsm/kg), $MgSO_4$ (avg. 684±42.8 mOsm/kg), urea (avg. 739±52.6 mOsm/kg), $CaCl_2$ (avg. 758±32.4mOsm/kg) and glucose (avg. 762±35.0 mOsm/kg) (Fig. 2). A one-way ANOVA revealed differences between solutions (F=10.194, df=5,20, P<0.0001) with KCl activating at a lower osmolality than $CaCl_2$ (Tukey's HSD, P=0.007), glucose (Tukey's HSD, P=0.006) and urea (Tukey's HSD, P=0.003). In addition, NaCl activated at a lower osmolality than $CaCl_2$ (Tukey's HSD, P=0.007), glucose (Tukey's HSD, P=0.005) and urea (Tukey's HSD, P=0.001), while the activation level of $MgSO_4$ was not different than any other solution (Tukey's HSD, P=0.216-0.307).

2.3.2. Linearity

At 1050 mOsm/kg all solutions activated sperm with a linearity of 0.90 or higher with the exception of $CaCl_2$ that produced an average linearity of 0.59±0.06 (Fig. 3). This visually correlated with the sperm track paths that were circular for $CaCl_2$ but relatively straight for all other solutions (Fig. 4).

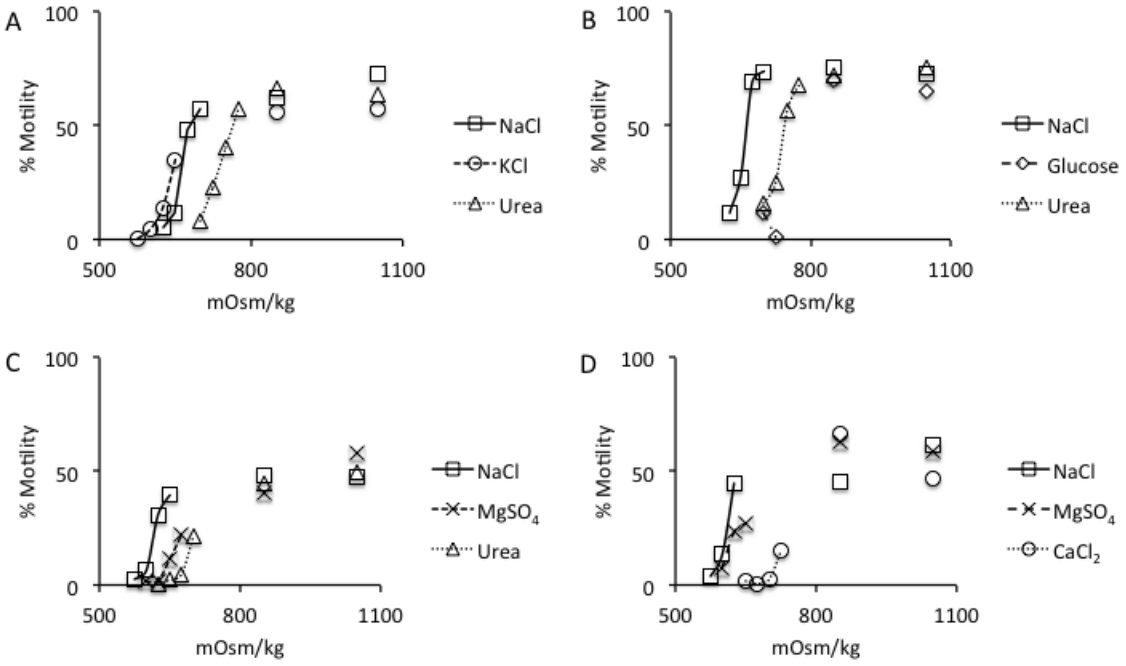

Figure 1. Representative results (one different male per panel) of the effects of KCl and urea (A) urea and glucose (B) $MgSO_4$ and urea (C) and $MgSO_4$ and $CaCl_2$ (D) all relative to NaCl on sablefish sperm motility.

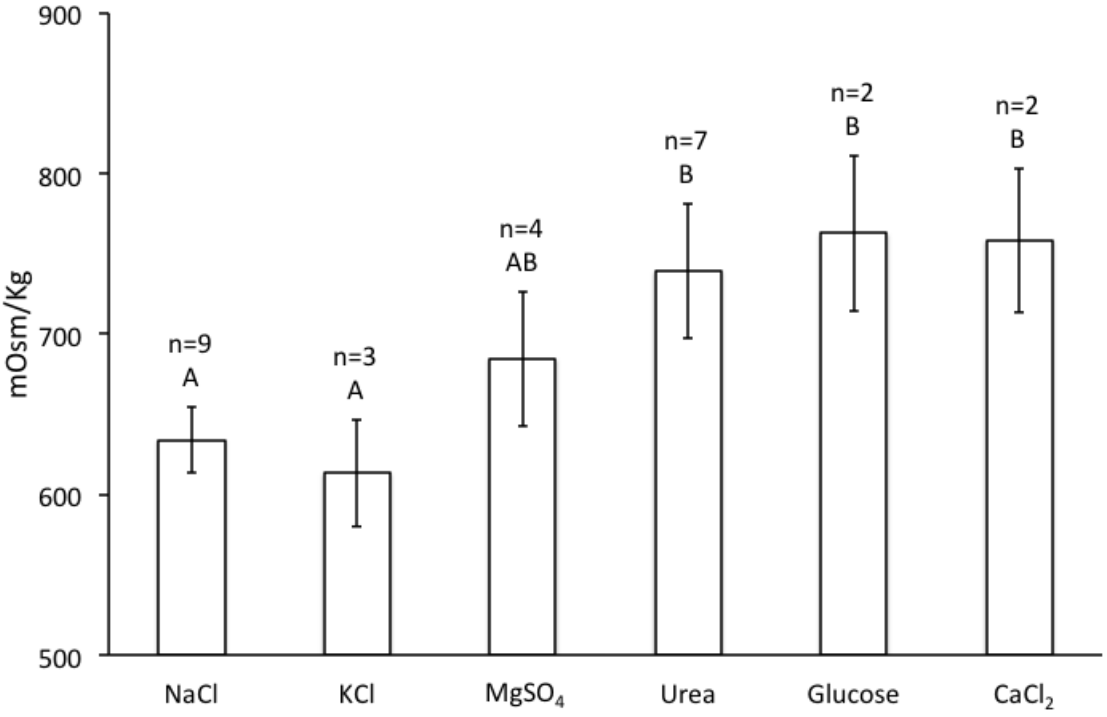

Figure 2. The average 50% activation value ± 95% confidence intervals for sablefish sperm activated with NaCl, KCl, $MgSO_4$, urea, glucose and $CaCl_2$ at various osmolalities (mOsm/kg). Bars not sharing the same letter are significantly different (P<0.05).

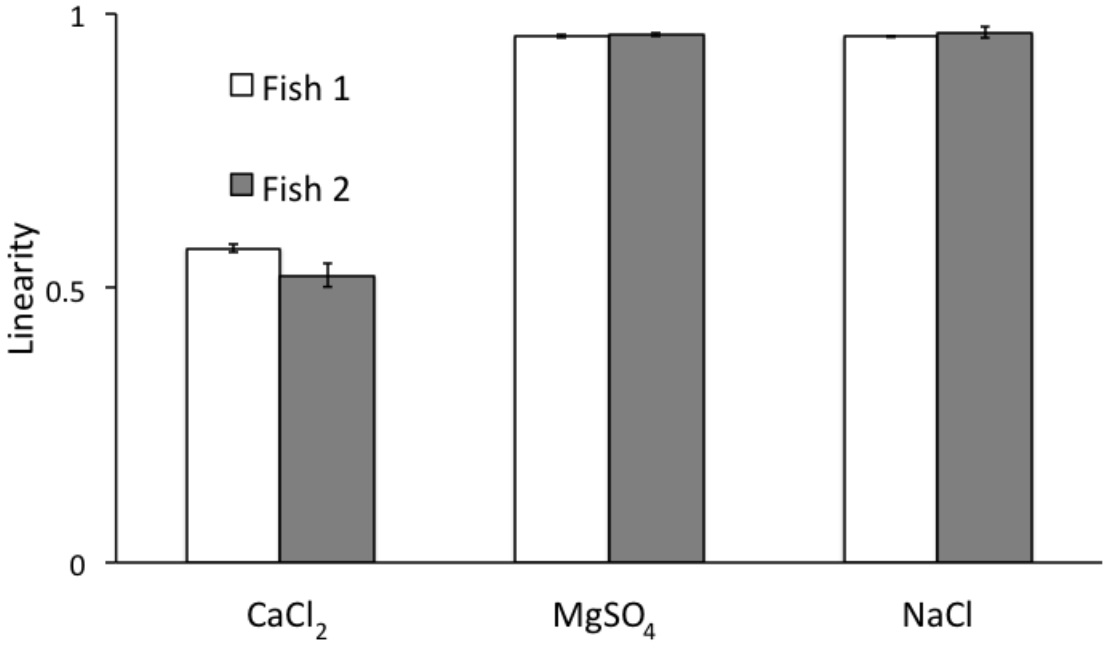

Figure 3. Linearity of sablefish sperm activated by $CaCl_2$ and NaCl. The closer the linearity is to 1.0, the straighter the sperm path.

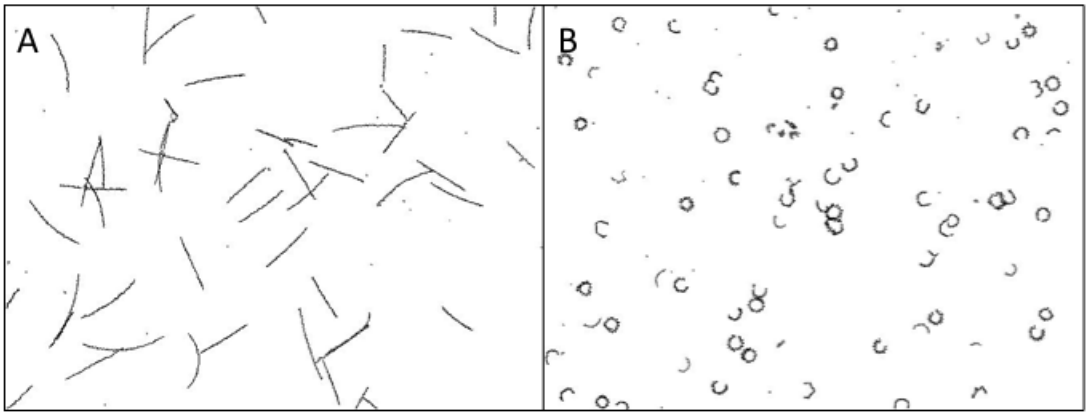

Figure 4. Sablefish sperm paths recorded over a 1 second duration following activation by A) 1050 mOsm/kg NaCl and B) 1050 mOsm/kg $CaCl_2$ at 10x magnification.

2.3.3. Cryopreservation

For percent motility (Fig. 5), a three-factor ANOVA revealed differences between the cryoprotectants (F=98.749, df=2,36, P<0.0001), cryoprotectant concentrations (F=76.285, df=1,36, P<0.0001) and freezing rates (F=4.249, df=2,36, P=0.02). Significant interactions were observed with cryoprotectant and freezing rate (F=5.674, df=4,36, P=0.001) and between cryoprotectant and concentration (F=11.081, df=2,36, P=0.0001). There were differences in motility observed between all cryoprotectants (Tukey's HSD, P<0.0001), the 5% and 10% cryoprotectant concentrations (Tukey's HSD, P<0.0001), and between the 2.5°C/m and 7.5°C/m freezing rate (Tukey's HSD, P=0.018). The overall motility maintained following freezing was different for all treatments containing DMSO at a concentration of 10% at all freezing rates (Tukey's HSD, P=0.001-0.011), compared to other cryoprotectant treatments with the exception of DMSO (5°C/min and 7.5°C/min) compared to 10% glycerol (2.5°C/min and 7.5°C/min) (Tukey's HSD, P=0.083-0.182).

A three-factor ANOVA for curvilinear velocity indicated differences only between cryoprotectants (F=4.132, df=2,43, P=0.023). Of the three cryoprotectant comparisons only the glycerol-propylene glycol were different (Tukey's HSD, P=0.016). For every cryoprotectant there were treatments that had curvilinear velocities that were the same as pre-cryopreservation values (Dunnett's P=0.058-0.671), as well as treatments that had lower curvilinear velocities (Dunnett's P=0.001-0.046) (Fig. 6).

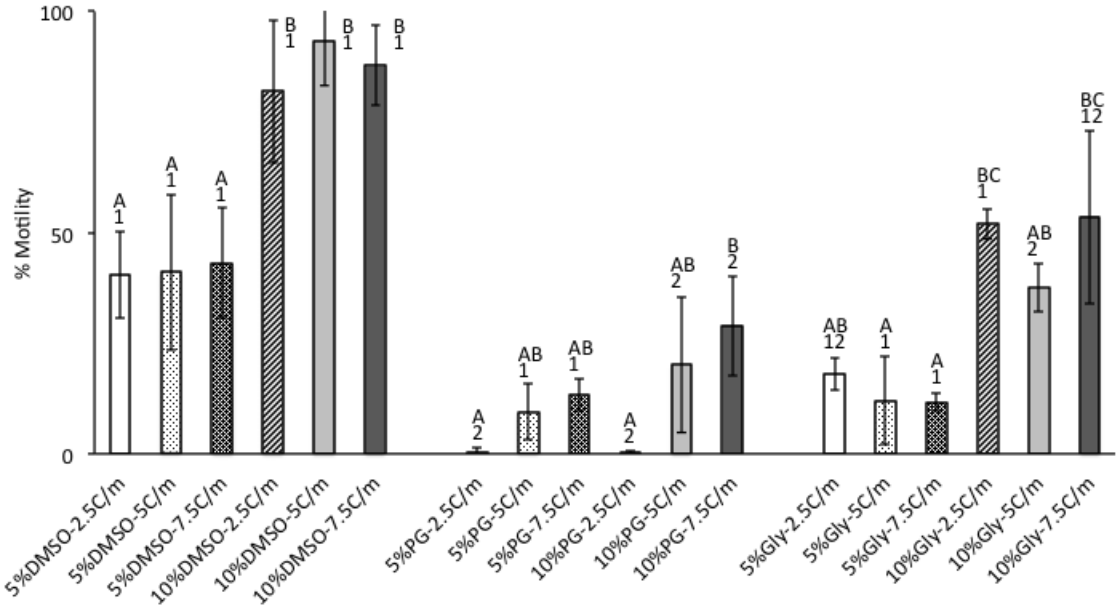

Figure 5. The % motility maintained of sablefish sperm post thaw (± SD) for dimethyl sulfoxide (DMSO), propylene glycol (PG), and glycerol (Gly) at concentrations of 5% and 10%, and freezing rates of 2.5°C, 5°C, or 7.5°C per min (C/m). Bars not sharing the same letter are significantly different (P<0.05) for a given cryoprotectant. Bars not sharing the same number are significantly different (P<0.05) for a specific treatment (concentration and freezing rate) across cryoprotectant.

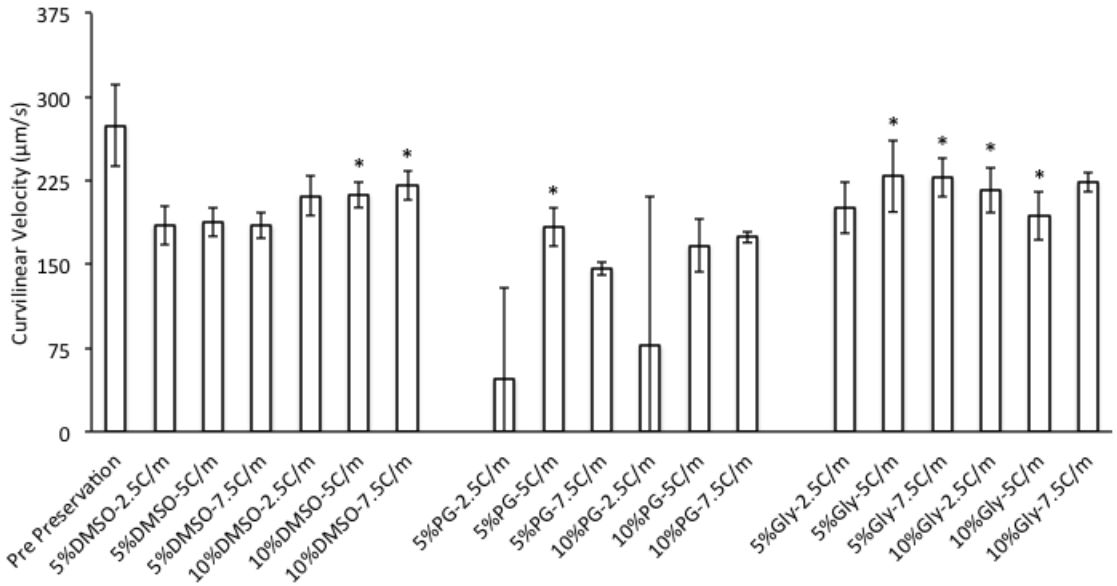

Figure 6. The curvilinear velocity of sablefish sperm (µm/s) ± SD before cryopreservation and after cryopreservation for dimethyl sulfoxide (DMSO), propylene glycol (PG), and glycerol (Gly) at concentrations of 5% and 10%, and freezing rates of 2.5°C, 5°C, or 7.5°C per min (C/m). Bars with an * are not significantly different (P>0.05) from pre-cryopreservation levels.

2.4.0. Discussion

In the present study, we clearly show that a hypertonic external environment is the most important stimulus for sablefish sperm activation, and that activation is not based on the presence or lack of a specific ion. Different species of fish rely on a variety of mechanisms for sperm activation. In seawater acclimated tilapia (*Oreochromis mossambicus*) sperm activation requires $CaCl_2$, while species from the family Cyprinidae exhibit motility in response to low osmotic pressure (Linhart et al. 1999; Alavi & Cosson 2006). Further, in some species K^+ acts as an activation inhibitor. However, this is most often noted in species from Salmonidae and Acipenceridae (Cosson 2004) and does not seem to apply to sablefish sperm since activation was not adversely affected when KCl was used as an activating medium. Activation is initiated by hypertonicity in some marine species such as Atlantic cod (*Gadus morhua),* and by the presence of calcium in some freshwater species such as trout (Cosson 2004; Alavi & Cosson 2006; Westin & Nissling 1991). Generally marine fish sperm is activated thorough an external osmotic rise. For example, the optimal range of osmolality for the activation of halibut (*Hippoglossus stenolepis)* is 900-1100 mOsm L^{-1} (Billard et al. 1995) while turbot (*Scophthalmus maximus)* sperm can be activated in hypertonic sugar solutions (Chauvaud et al. 1995).

All hypertonic solutions tested were able to activate sablefish sperm. NaCl and KCl activated at lower osmolalities than did $CaCl_2$, urea and glucose. This indicates that sablefish sperm can be activated by hypertonicity alone but is enhanced by some ionic solutions such as KCl or NaCl, especially at a lower osmolality. Activation was not observed when sperm was diluted with various ionic and nonionic iso-osmotic solutions,

dispelling any possibility that specific ions play an role in inhibitory role on activation. In the present study, non-ionic solutions like glucose and urea required a much higher osmolality for activation compared to ionic solutions such as NaCl and KCl. The only ionic solution that required a higher osmolality was $CaCl_2$. While hypertonicity is the primary stimulus for sablefish sperm activation, there are clearly additional effects of the type of solution as well as effects of different ions in the solution.

In marine fish sperm that can be activated by hypertonicity alone, it is understood that an increase in osmolality causes an osmotic exit of water from the cell through local aquaporins, causing membrane stretching (Cosson et al. 2008). The stretching of the membrane triggers a response by stretch-activated channels, thus increasing local permeability allowing for increased ion conductivity through channels and for water to move rapidly through porins (Cosson et al. 2008; Yang & Sachs 1993). Activated aquaporins are capable of increasing water permeability up to three fold. The increase of the internal ionic concentration initiates an auto-catalytic effect along the flagellar membrane thus self-amplifying the ionic concentration effect. The increased internal ionic concentration that occurs through the rapid pumping of water out of the cell and transport of ions into the cell, causes ionic concentrations to reach optimal levels for the dynein to act as a motor, thus initiating flagellar beating (Cosson et al. 2008).

With activation by high osmolality KCl or NaCl solutions, high concentrations of Na^+ and K^+ outside the cell would increase the internal ionic concentration faster than other solutions because Na^+ and K^+ would move into the cell through open channels while water is moving out. K^+ has a slightly greater effect than Na^+ because the relative permeability of K^+ through the cellular membrane is 3-4 fold greater than that of Na^+

(Kaupp et al. 2008). Non-ionic solutions did initiate motility but at higher osmolalities than KCl or NaCl. This is most likely due to the fact that changes in ionic concentration in the cell must occur as a consequence of water transport alone without the aid of an additional influx of ions into the cell. $MgSO_4$ was more effective than non-ionic solutions but less effective than KCl and NaCl. Thus, Mg^{2+} may be able to enter the cell but the membrane may not be as permeable to it as it is to K^+ or Na^+.

In the current study, $CaCl_2$ required a much higher concentration to induce activation than other ionic solutions and also initiated a chemotactic response exemplified by low linearity and circular motion of the sperm. Peptides released from eggs to induce chemotaxis have been well studied in a number of marine invertebrates (Kaupp et al. 2006; Ward & Kopf 1993). This response has also been noted in aquatic vertebrates such as Atlantic salmon (*Salmo salar),* brown trout (*Salmo trutta)* and Pacific herring (*Clupea pallasii)* (Yeates et al. 2013; Morisawa 2008). Chemotaxis decreases the linearity of the sperm's swimming path by decreasing flagellar symmetry. These peptides typically stimulate motility, and the metabolism of phospholipids while increasing oxygen consumption, cAMP, cGMP, Ca^{2+}, and pH (Ward & Kopf 1993). Ca^{2+} changes flagellar beat symmetry by acting on the axoneme (Brokaw 1991). The binding of oligopeptides to the receptor guanylyl cyclase initates a cascade of events that lead to a hyperpolarization of the cellular membrane and the opening of calcium channels (Kaupp et al. 2008). This signaling uses the Ca^{2+} that is prevalent in seawater. Thus, the activation response observed in sablefish sperm to higher $CaCl_2$ solutions was most likely a result of the influx of Ca^{2+} into the cell that could have mimicked a naturally-induced chemotactic response.

Methods were also developed to cryopreserve sablefish sperm, and the most successful treatment appears to include 10% DMSO. A number of cryoprotectants have been used to cryopreserve sperm of a variety of fish species. The most successful cryoprotectant tested for sablefish milt, DMSO, is a very popular cryoprotectant for use in animal cells and has been used successfully at concentrations ranging from 5-15% for the cryopreservation of sperm of a number of fish species, including Arctic char (*Salvelinus alpinus,* Richardson et al. 2000), barramundi (*Lates calcarifer,* Leung 1987), grouper (*Epinephelus tauvina*, Withler & Lim 1982), Atlantic croaker (*Micropogonias undulatus,* Gwo et al. 1991) and yellowtail flounder (*Pleuronectes ferrugineus,* Richardson et al. 1995). However, the success of cryoprotectants may be species-specific since glycerol has offered better protection than DMSO in European catfish (*Silurus glanis*, Linhart et al. 1993), summer flounder (*Paralichthys olivaceus,* Zhang et al. 2003), and yellowfin seabream (*Acanthopagrus latus,* Gwo 1994) sperm. In contrast, glycerol failed to offer any protection to black grouper sperm (*Epinephelus malabaricus*, Gwo 1993) and for sablefish it offered very little protection compared to DMSO. Propylene glycol offered low to marginal protection for summer flounder (Zhang et al. 2003) and sablefish (present study) sperm, but successfully protected winter flounder (*Pseudopleuronectes americanus,* Rideout et al. 2003), haddock (*Melanogrammus aeglefinus)* and Atlantic cod (Rideout et al. 2004) sperm.

Optimal freezing rate can also be very species-dependent. For marine fish species, optimal freezing rates vary significantly from 5°C/min for cod (Mounib et al. 1968) and 8°C/min for hirame (*Paralichthys olivaceus,* Tabata & Mizuta 1997) to 99°C/min for turbot (Dreanno et al. 1997) with many species falling in between. The freezing rates

tested for sablefish were all on the slow end for freezing rates in general, but these rates did not have a deleterious effect on the cryopreservation of sablefish sperm.

Curvilinear velocities were lower for some treatments for every cryoprotectant and seemed to correlate with low percent motility. However, this was not always the case and there were some instances where low percent motility was associated with normal curvilinear velocities. This shows that even in treatments that induced lower percent motility, the curvilinear velocities of the motile sperm could still be maintained.

In conclusion, sablefish sperm was effectively activated by hypertonic solutions in general, and to the greatest extent by ionic solutions such as NaCl and KCl. In addition, good motility could be maintained in sablefish sperm cryopreserved with 10% DMSO at all of the freezing rates tested.

CONCLUSIONS

Sablefish are a commercially important species, yet this study is the first to investigate the stimulus for sablefish sperm activation and to determine the requirements for successful cryopreservation.

- The primary stimulus for sablefish sperm activation was a rise in external tonicity. Presumably this hypertonicity caused an increase in the intracellular ionic concentration, in turn, initiating flagellar movement.

- Compared to other solutions tested, the activation response could be initiated at lower osmolalities with solutions containing K^+ or Na^+. Both of these ions are able to pass through the membrane of the sperm cell in fishes and accentuate the increase of the ionic concentration within the cell.

- Increasing concentrations of $CaCl_2$ activated sablefish sperm, but only at concentrations higher than NaCl or KCl, and produced circular sperm paths typical of a chemotactic response.

- Cryopreservation of sablefish sperm was sensitive to cryoprotectant type (DMSO, glycerol, propylene glycol) and the best retention of motility following freezing was accomplished using 10% DMSO at freezing rates of -2.5, -5 and -7.5 °C/min.

- Curvilinear velocity could be maintained using any cryoprotectant tested, and was not dependent on cyroprotectant type or freezing rate.

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