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Cryopreservation spermiation induction and computer assisted analysis of summer flounder (Paralicthys dentatus) spermatozoa

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CRYOPRESERVATION, SPERMIATION INDUCTION AND COMPUTER ASSISTED ANALYSIS OF SUMMER FLOUNDER (*PARALICTHYS DENTATUS*) SPERMATOZOA

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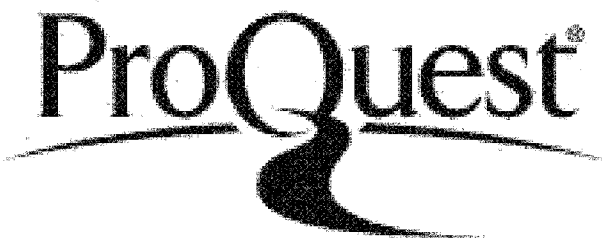

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

CRYOPRESERVATION, SPERMIATION INDUCTION AND COMPUTER ASSISTED ANALYSIS OF SUMMER FLOUNDER (*PARALICTHYS DENTATUS*) SPERMATOZOA

by

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The objective of this thesis was to improve the aquaculture practices for summer flounder *Paralichthys dentatus*. The experiments in this thesis examined male reproduction. First a practical method for summer flounder sperm cryopreservation was developed. These experiments examined the various parameters required to successfully freeze and store summer flounder spermatozoa. Success of cryopreservation was measured by examining post thaw viability and fertility.

One of the problems facing summer flounder aquaculture is sexually dimorphic growth rates, with females growing larger than males. To overcome this, meiogynogenetic summer flounder were developed, allowing the production of all female stocks. The second set of experiments examined the sperm characteristics and fertility of meiogynogentic summer flounder sperm. Finally, to overcome small milt volumes in summer flounder the final set of experiments examined the used of exogenous hormones to increase summer flounder sperm production.

INTRODUCTION

In the last decade, the global capture fisheries have plateaued with as many as 50% of the world's commercial fish stocks at or beyond sustainable harvests (FAO, 2008). Locally, the northeast ground fisheries, once considered to be among the most productive in the world, have declined steadily due to overfishing and environmental changes with devastating consequences to New England fishing communities (NMFS, 2002). The decline in wild fisheries has coincided with a greater worldwide demand for seafood, and the increased demand has been met entirely through increased aquaculture production. From 2004-2006 aquaculture grew at an annual rate of 11% in value, and 6.1% in volume. Currently, aquaculture continues to grow faster than any other animal producing sector, (8.9% annually) but much of this growth is occurring in Asia, and China in particular (FAO, 2008).

The United States is a major consumer of marine aquaculture products, yet we grow only a small fraction of what we consume. In recent years, the US trade deficit associated with seafood ranked second behind oil and is estimated in excess of $9 billion in 2000 (ARS 2003). Our reliance on imported aquaculture products in some cases promotes environmental degradation in countries with less stringent regulations. A major obstacle to the growth of a U.S. aquaculture industry has been the need to find environmentally sustainable methods of farming fish.

Although simple forms of aquaculture have been practiced for thousands of years, intensive, commercial aquaculture only began in the 1960s. This burgeoning industry faced many challenges as it has shifted from small-scale, extensive production of

herbivorous and omnivorous species, often grown in freshwater ponds, to intensive production of marine finfish and shrimp. One problem that the industry faced was developing cost-effective feeds because feed costs are considered to be the highest recurrent expenditure in an aquaculture enterprise. This single expense can account for 60% of the operating budget for highly intensive culture of carnivorous fish (DeSilva and Anderson, 1995). Protein, as the most expensive nutrient in aquaculture feeds, contributes significantly to costs, especially in carnivorous fish. Protein in feed pellets is typically derived from fishmeal that is made from wild harvested stocks of economically less valuable species (primarily anchovies). Pellets used for marine finfish aquaculture typically contain up to 40% fishmeal, which provides protein (~70% of fishmeal) and fat (~10% of fishmeal) to the diets. It has been estimated that the aquaculture industry currently consumes 34% of the global fishmeal production and will consume 50% by the year 2013 (Naylor et al., 2000; Deutsch et al., 2006). The fishmeal market is highly unstable with changing year-to-year availability and significant price fluctuations (Rondán et al., 2004). Due to its relatively high cost, cost variability, and growing environmental concern about harvesting wild fish to produce fishmeal, it is desirable to replace fishmeal with less expensive protein sources (Tidwell et al., 2005). Partial or full fishmeal replacement using proteins derived from plant sources has proven effective for many teleost species (Carter and Hauler, 2000).

In addition to feed ingredients, the aquaculture industry has also faced criticism for contributing to environmental degradation and disease transmission to wild fish. Finfish in many regions are grown in near-shore net pens, often in high densities.

Culturing animals in cages or net pens, however, can degrade water quality through waste accumulation. Growing fish in high densities can also increase the prevalence of pathogens. In recent years the Atlantic salmon industry has had to contend with outbreaks of infectious salmon anemia, caused by a virus (*Isavirus*) and fish lice (*Lepeophtherius salmonis*), an external parasitic crustacean (Berg and Hornsberg, 2008). Because of these problems, there is considerable interest in developing cost-effective, land-based systems that reduce or eliminate interactions with the external environment.

Intensive, land-based systems that continuously re-use water (recirculating aquaculture (RA) systems) allows for complete control of environmental conditions, which permits optimal growth and performance while eliminating environmental impacts. For the most part, the high operational and construction costs of land-based, RA systems have necessitated culturing fish that can be cultured at extremely high densities, in order to recover the infrastructure and operational costs (economy of scale). Alternatively, very high-valued species (e.g., ornamentals) have been grown successfully at lower densities. Because of recent developments in RA technology, and greater understanding of the culture requirements of some high valued, marine food fish, however, the economics associated with RA culture have changed considerably. Compared to earlier iterations of recirculating systems that required up to 10% daily water replacement and resulted in metabolite (nitrate) accumulation, new generation RA systems can operate at or near 100% efficiency (no water loss) with nearly complete metabolite elimination. These design changes have lead to the development of several commercial-scale, land-based aquaculture operations for foodfish in the northeast, including Local

Oceans™ (NY) and Australis (Turners Falls. MA). The selection of appropriate species for RA systems is of the upmost importance and is dependent on a number of factors such as market value, ease of domestication, and time required to reach market size (Webber and Riordan et al., 1975).

In the U.S., considerable research has been conducted to determine practices conducive for the culture of summer flounder (*Paralichtys dentatus*) (Bengtson 1999; Burke et al., 1999; Watanabe and Carroll, 2001; Gaylord et al., 2004; Veillette et al., 2007). Summer flounder are a high-value species and fishing pressure has significantly reduced wild catches, such that demand often exceeds supply (NOAA, 2008). Research on summer flounder aquaculture began in 1970, however, due to a lack of commercial interest and funding, culture of summer flounder was not initiated until 1990. The framework of summer flounder aquaculture was modeled after that of Japanese flounder (*Paralicthys olivaceus*) from Asia, and Turbot (*Psetta maxima*) from Europe (Bengston, 1999). Summer flounder aquaculture began in part because of a decline in commercial landings from a peak of 18,000 metric tons (mt) in 1980 to 4,143 mt in 2008 (NOAA, 2009). Growth rate remains the dominant factor controlling profitability of land-based culture of this species, and the costs associated with juvenile growth to market size must be reduced to gain competitiveness on the global market (King et al., 2001). Summer flounder, like other Paralichthid species, exhibit sexually dimorphic growth rates, with females growing considerably faster and larger than males (Morse, 1981; King et al., 2001). A tremendous increase in growth performance can therefore be realized through the production of all-female populations of fingerlings. Monosex production of teleost

populations has been accomplished by several mechanisms, including chromosome manipulation (polyploidy), gynogenesis and exogenous steroid production (Wolters et al., 1982; Benfey and Sutterlin, 1984). Monosex populations of Japanese flounder (*P. olivaceus*) have been produced commercially in Japan and Korea since 1990 and 1995, respectively (Seikai, 2000; Yamamoto, 1999)

Meiogynogenesis

Diploid gynogenesis has been accomplished for several fish species, including summer flounder (Colburn et al., 2009) southern flounder (*P. lethostigma*; Luckenbach, et al., 2004; Morgan et al., 2006), and Japanese flounder (Tabata et al., 1986), and involves a two-step process. Initially, oogenesis is reinitiated by fertilizing eggs with genetically-inactivated, but motile spermatozoa. Ultraviolet (UV) irradiation has been successfully used in a number of fish species to crosslink paternal DNA and produce genetically-inactivated sperm (Morgan, et al., 2006). The use of untreated or UV-irradiated, heterologous sperm further ensures that no parental genetic contribution is possible and that all surviving larvae are produced by gynogenesis. The second step involves re-establishing diploidy by blocking expulsion of the second polar body (meiotic gynogenesis or meiogynogenesis) or preventing the first embryonic cell division (mitotic gynogenesis or mitogynogenesis) with the use of thermal or physical shock shortly after fertilization (Ihssen et al., 1990). These procedures, coupled with exposure to proper sex-determining environmental conditions, have been used to sex-reverse broodstock of several important aquaculture species for production of monosex populations (Devlin and Nagahama, 2002; Ihssen, et al., 1990; Morgan, et al., 2006). Summer flounder

meiogynogens were sex reversed by exposing them to high water temperatures (>21°C; Colburn et al. 2009) and sperm from these individuals were used in the experiments described in Chapter II.

Gametogenesis

Controlled reproduction and high quality gamete production are essential for profitable aquaculture to ensure adequate availability of larvae and juveniles (Mylonas et al., 2010). In temperate fish species, reproduction is controlled by seasonal changes in water temperature, day length, availability of nutrients (Sarkar et al., 2010), and social stimuli (Stacey et al., 2001). These environmental stimuli stimulate reproduction by their influence on the hypothalamo-pituitary-gonadal axis. The environmental cues stimulate the hypothalamus to release gonadotropin releasing hormones (GnRHs) which bind to receptors on gonadotropes in the anterior pituitary gland. Once stimulated by GnRHs, the gonadotropes synthesize and secrete gonadotropins (Gths), luteinizing hormone (LH) and follicle stimulating hormone (FSH) that subsequently stimulate steroidogenesis by follicle cells in the gonads (Mylonas et al., 2010). In cultured fish, these processes may be disrupted by stressors associated with captivity, and/or improper environmental conditioning. To reduce reproductive dysfunction in cultured fish, husbandry practices should minimize stress and environmental cues should simulate natural conditions (Schreck et al., 2001).

For some species, such as Atlantic cod (*Gadus morhua*), larvae are typically obtained from group, volitionally spawning broodstock housed in large tanks (tank spawning). Under this system no genetic selection can be applied, as parentage is

unregulated. Environmental cues, most notably photoperiod and water temperature, regulate reproductive development, but the date of spawning initiation can vary by several weeks from one year to the next.

An alternative to tank spawning is strip spawning where fully developed gametes are manually expressed from selected individuals for *in vitro* fertilization. *In vitro* fertilization is often employed with species that fail to spawn volitionally in captivity, and to control parentage for genetic improvement and manipulation of ploidy conditions. Additionally, tank spawning is often prolonged over a period of weeks, which extends the labor-intensive larviculture period and the need for algal and live-feed production. *In vitro* fertilization synchronizes and consolidates spawning efforts.

Despite optimal husbandry, broodstock of some species fail to undergo proper gametogenesis, resulting in poor gamete quality, and/or fail to release mature gametes (ovulation and spermiation in females and males, respectively). In such cases, exogenous administration of Gths (or similar derivatives), GnRHs or extracts of freeze-dried pituitaries (carp, salmon) have proven beneficial to alleviate reproductive failure in many species. The specific treatments are species-specific, must be determined experimentally, and in general have been found more effective for inducing ovulation than spermiation (Mylonas and Zohar, 2001).

Gametogenesis in males occurs in two phases. Spermatogonia replicate by mitosis and undergo two meiotic divisions to form four spermatids. Spermiation occurs during the spawning season resulting in the release of seminal plasma and spermatozoa from the testes. In a culture setting expelling milt from the testes can be accomplished by gentle

abdominal massage or stripping milt from a ripe male (Mylonas et al., 2010). Spermatogenesis may be continuous in fish species with tubular testes, or discontinuous in species with lobular testes (Vizziano et al., 2008). In male fish, spermatogenesis is under gonadotropic control, specifically, FSH dominates during spermatogenesis and LH during spermiation. LH is also responsible for stimulating androgen production in Leydig cells, located between the seminiferous tuble and the interstitial area of the testes. FSH stimulates Sertoli cell proliferation, for which germ cells are dependent for survival and development (Schulz and Miura, 2002).

Cryopreservation

Like many other domesticated animals, methods to preserve sperm by freezing have been developed for many species of commercially important and threatened fishes. The ability to cryopreserve sperm from brood stock has many benefits including synchronized gamete availability for *in vitro* fertilization, preservation of genetic material from superior brood stock, transportation of spermatozoa among hatcheries, conservation of genetic material from threatened fish species, sperm economy in species where very low volumes are produced, and minimizing brood stock maintenance. (Billard, 1986; Suquet et al., 2000; Cabrita et al., 2010). There have been research efforts to develop cryopreservation methods for paralichthid species such as the Japanese flounder (*Paralicthys olivaceus)* and Brazilian flounder (*Paralichthys orbignyanus*) (Zhang et al., 2003; Lanes et al., 2008), but to date no methods have been developed for summer flounder.

Successful sperm cryopreservation with high post-thaw viability is dependent on several factors including sperm quality prior to freezing, freezing rate, extender composition, cryoprotectant type and concentration, and extent of sperm dilution (Rana, 1995; Gwo, 2000; Chao and Liao, 2001; Yang and Tierch, 2009). No combinations of cryoprotectant and extender are universal, so sperm cryopreservation methods must be catered to specific species (Routray et al., 2008). During freezing, sperm are susceptible to cold shock, osmotic stress, and intracellular ice formation (Cabrita et al., 2005). Further, mitochondria function can become compromised from freezing and thawing (Cabrita et al., 2005). Due to these problems, cryopreserved sperm often have lower fertility compared to fresh even after a cryopreservation technique has been optimized (Cabrita et al., 2005).

Initial sperm quality is highly variable, even among individuals of the same population and must be carefully evaluated prior to freezing (Rana, 1995). Sperm quality, even from individual fish can change chronologically, as intratesticular sperm aging has also been reported for many species, particularly at the end of the reproductive season (Susquet et al., 2000). Careful abdominal massage of sedated fish provides an effective, non lethal means of sperm collection for *in vitro* fertilization (Rana, 1995; Suquet, 2000). Contamination from water, urine, feces, and mucous during collection, however, can change the osmolality and pH of seminal fluid and further decrease sperm quality. Fecal and urine contamination of milt can be reduced by starving fish 6-24 hours and emptying their bladders prior to milt collection (Rana, 1995). Crushing dissected testes as a means of collecting sperm may greatly reduce the risks of contamination, however, the high

value of many brood stock make non-lethal means of sperm collection more desirable (Yang and Tierch, 2009). Because of the numerous biological factors and handling procedures that can affect post-thaw viability and fertilizing capacity, several methods have been developed to assess sperm viability prior to and following cryopreservation (Cabrita et al. 2005).

Sperm Evaluation

Spermatozoa motility is often used as an indicator of sperm quality, and the percentage of motile spermatozoa has been positively correlated with fertilization in many species (Levanduski and Cloud, 1988; Wang and Crim, 1998; Fauvel et al., 2010). In others, such as Atlantic cod, *Gadus morhua* L., (Trippel and Neilson, 1992); salmonids (Scott and Baynes, 1980), and rosy barb, *Barbus conchonius* (Amanze, 1994) fertilization success using *in vitro* fertilization was not different using motile or immotile sperm. Differential staining of live and dead cells, based on their membrane integrity, has proven useful for rapid evaluation of preservation protocols prior to fertilization trials (DeGraaf and Berlinsky, 2004; Lanes et al., 2008; Cabrita et al., 2009). The recent use of fluorescent dyes such as SYBR-14 and propidium iodide, in combination, permits fast and accurate viability analysis. (Cabrita et al., 2005). Cells with intact, selectively permeable membranes restrict passage of propidium iodide and SYBR-14 stains their nucleic acids green. Cells with compromised membranes do not restrict propidium iodide and cells stain red (Horváth et al 2006). These stains were incorporated into a kit for staining sperm cells (Live/dead® sperm viability kit) that was used to assess cell viability in the studies described in Chapter 1.

Other methods of assessing post thaw spermatozoa viability include assays that examine mitochondria functionality. This can be accomplished using the lipophillic cation JC-1 and flow cytometry. JC-1 changes from orange to green when mitochondrial membrane potential is low (Cabrita et al., 2005).\

Extenders

Diluting sperm with an extender solution is essential prior to cryopreservation (Rana,1995; Yang and Tierch, 2009). During freezing and thawing, biological salts may lose their buffering capacity and extender solutions play an important role in maintaining osmolality and pH (Yang and Tierch, 2009). In the spermatozoa of marine fishes, exposure to hypertonic solutions causes motility activation (Morisawa and Suzuki, 1980) and selected extenders must be iso-osmotic to the seminal fluid, to ensure it does not cause spermatozoa activation (Rana, 1995). In addition to buffering capacity, suitable extenders contain nutrients, stabilizing colloids, and antioxidants. (Gwo, 2000).

Cryoprotectants

By adding a cryoprotecting agent to extended sperm it is possible to prevent the formation of intercellular ice crystals and cellular dehydration during cryopreservation (Yang and Tierch, 2009). Effective cryoprotectants should be highly soluable, able to penetrate cell membranes easily and minimally toxic to spermatozoa (Suquet et al., 2000; Chao and Liao, 2001; Yang and Tierch, 2009). Common cryoprotectants used include dimethyl sulfoxide (DMSO), glycerol, ethylene glycol and methanol. Glycerol has been widely used for sperm cryopreservation of salmonid and marine species (Suquet et al., 2000) but because it is slow to penetrate membranes, it requires an equilibration period,

that increases toxicity. For that reason, glycerol has been replaced by DMSO for sperm cryopreservation of many species. While DMSO has proven widely successful for a number of marine teleosts, and has been called the "universal cryoprotectant" (Chao and Liao 2001), equal or greater success has been attained with other cryoprotectants. For instance, compared to DMSO, greater success was attained using glycerol, propylene glycol, and trehalose as sperm cryoprotectants for Japanese flounder, winter flounder, *Pseudopleuronectes americanus* (Walbaum), and orange-spotted grouper, *Epinephelus coioides* (Hamilton), respectively (Rideout et al., 2003; Zhang et al., 2003; Peatpisut and Amrit, 2010).

Freezing rates and methodology

Many packaging vessels and freezing systems have been successfully employed for teleost sperm cryopreservation. Packaging vessels such as plastic straws, cryovials and glass capillary tubes have been used successfully and their specific use often depends on the species-specific sperm volumes required. Some freezing protocols have also been employed using dry ice, liquid nitrogen and controlled rate freezers (Stoss and Donaldson, 1983; Susquet et al., 2000). The range of optimal freezing rates varies between -1° C/minute to -99 ° C/minute (Susquet et al., 2000). Highly successful sperm cryopreservation has been conducted using a two-step method, during which sperm are initially held in liquid nitrogen vapors for some period and then plunged directly into the liquid nitrogen for storage. Cooling rate is determined by the height samples are placed above the vapor. Problems with this method may can arise due to inconsistency of sample

placement. These problems are avoided by using a programmable controlled-rate freezer, as was employed in the studies described in Chapter 1.

Short term storage

Short term storage of fish spermatozoa is useful when sperm and eggs may be collected at different times or locations. The purpose of short term milt storage is to slow metabolic activity of spermatozoa so their life span may be extended. Most fish sperm are suitable for storage because they remains quiescent in the seminal plasma, and since they remain immotile they consume less energy. Because of this characteristic fish spermatozoa may be stored for hours or even days and still remain viable. Problems that may reduce the viability during short term storage of spermatozoa include inadequate temperature control or gaseous exchange, bacterial contamination and cell desiccation. These obstacles may be overcome by the addition of a diluent and/or gaseous oxygen to the spermatozoa (Gwo, 2000; Rana, 1995).

References

Amanze D., 1994. Strategies for sperm-egg contact during external fertilization in teleosts. High performance fish: Proceedings of an International Fish Physiology Symposium at the University of British Columbia in Vancouver, Canada. July 16-21, pp. 176-181. Fish Physiology Association,Vancouver, BC, Canada.

ARS, U., 2003. ARS National Programs. http://www.ars.usda.gov/research/programs/programs.htm?np_code=106&docid=832.

Benfey T.J., Sutterlin A.M., 1984. Growth and gonadal development in triploid landlocked Atlantic salmon (*Salmo salar*). Canadian Journal of Fisheries and Aquatic Sciences 41, 1387-1392.

Bengtson D.A., 1999. Aquaculture of summer flounder (*Paralichthys dentatus*): Status of knowledge, current research and future research priorities. Aquaculture 176, 39-49.

Berg A.G.T., Horsberg, T.E., 2008. Plasma concentrations of emamectin benzoate after Slice(TM) treatments of Atlantic salmon (*Salmo salar*): Differences between fish, cages, sites and seasons. Aquaculture 288, 22-26.

Billard R., 1986. Spermatogenesis and spermatology of some teleost fish species. Reproduction, Nutrition and Development 26, 877-920.

Burke J.S., Seikai T., Tanaka Y.,Tanaka M., 1999. Experimental intensive culture of summer flounder, *Paralichthys dentatus*. Aquaculture 176, 135-144.

Cabrita E., Robles V., Rebordinos L., Sarasquete C., Herraez M.P., 2005. Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. Cryobiology 50, 144-153.

Cabrita E., Sarasquete C., Martinez-Páramo S., Robles V., Beirão J., Pèrez-Cerezales S., Herráez M.P., 2010. Cryopreservation of fish sperm: applications and perspectives. Journal of Applied Ichthyology 26, 623-635.

Carter, C.G., Hauler, R.C., 2000. Fish meal replacement by plant meals in extruded feeds for Atlantic salmon, *Salmo salar* L. Aquaculture 185, 299-311.

Chao, N.H., Liao, I.C., 2001. Cryopreservation of finfish and shellfish gametes and embryos. Aquaculture 197, 161-189.

Colburn, H.R., Nardi., G.C., Borski, R.J., Berlinsky, D.L., 2009. Induced meiotic gynogenesis and sex differentiation in summer flounder (*Paralichthys dentatus*). Aquaculture 289, 175-180.

Cyr D.G., Eales J.G., 1996. Interrelationships between thyroidal and reproductive endocrine systems in fish. Reviews in Fish Biology and Fisheries 6, 165-200.

DeGraaf J.D., Berlinsky D.L., 2004. Cryogenic and refrigerated storage of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) spermatozoa. Aquaculture 234, 527-540.

De Silva S.S., Anderson T.A., 1995. Fish Nutrition in Aquaculture. Chapman & Hall Aquaculture Series, London. pp. 319.

Deutsch, L., Graslund, S., Folke, C., Troell, M., Huitric, M., Kautsky, N., Lebel, L., 2007. Feeding aquaculture growth through globalization: Exploitation of marine ecosystems for fishmeal. Global Environmental Change 17, 238-249.

Devlin, R.H., Nagahama Y., 2002. Sex Determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. Aquaculture 208, 191-364.

Donaldson E.M., Hunter G.A., 1983. Induced final maturation, ovulation, and spermiation in cultured fish. Fish Physiology: Vol. 9: Reproduction, Pt. B: Behavior and Fertility Control. Academic press. pp.351

FAO (2008) The State of World Fisheries and Aquaculture (SOFIA). FAO, Rome. Fischcher G, Shah M.

Fauvel C., Suquet M., Cosson J. 2010. Evaluation of fish sperm quality. Journal of Applied Ichthyology 26, 636-643.

Gaylord, T.G., Schwartz M.H., Cool R.W., Jahncke M.L., Craig S.R., 2004. Thermal optima for the culture of juvenile summer flounder, *Paralichthys dentatus*. Journal of Applied Aquaculture 14, 155-162.

Gwo J.C., 2000. Cryopreservation of sperm of some marine fishes. In: Cryopreservation in Aquatic Species (ed. by Tiersch T.R., Mazik P.M.) pp. 138-160, World Aquaculture Society, Baton Rouge, LA.

Habibi H.R., Marchant T.A., Nahorniak C.S., Van Der Loo H., Peter R.E., Rivier J.E.,Vale W.W., 1989. Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon gonadotropin-releasing hormones in the pituitary of goldfish (*Carassius auratus*). Biology of Reproduction 40, 1152-1161.

Horváth Á., Urbányi B., Mims S.D., Bean W.B., Gomelsky B., Tiersch T.R., 2006. Improved cryopreservation of sperm of paddlefish (*Polyodon spathula*). Journal of the World Aquaculture Society 37, 356-362.

Ihssen P.E., McKay L.R., McMillian I., Phillips, R.B., 1990. Ploidy manipulation and gynogenesis in fishes: cytogenetic and fisheries applications. Transactions of the American Fisheries Society 119, 698-717.

King N.J., Nardi G.C., Jones C.J. 2001. Sex linked growth divergence of summerflounder from a commercial farm: are males worth the effort? Journal of Applied Aquaculture 11, 77-88

Lam T.J., 1982. Applications of endocrinology to fish culture. Canadian Journal of Fisheries and Aquatic Sciences 39, 111-137.

Lanes C.F.C., Okamoto, M., Cavalcanti, P.V., Collares, T., Campos, V.F., Deschamps, J.C., Robaldo, R.B., Marins, L.F., Sampaio, L.A., 2008. Cryopreservation of Brazilian flounder (*Paralichthys orbignyanus*) sperm. Aquaculture 275, 361-365.

Le Gac F., Blaise O., Fostier A., Le Bail P-Y., et al., 1993. Growth hormone (GH) and reproduction: A review. Fish Physiology and Biochemistry 11, 1-6.

Lethimonier C., Madigou T., Munoz-Cueto J., Lareyre J., 2004. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. General and Comparative Endocrinology 135, 1-16.

Levanduski M., Cloud J., 1988. Rainbow trout (*Salmo gairderi*) semen: effect of non-motile sperm on fertility. Aquaculture 75, 171-179.

Luckenbach J.A., Godwin J., Daniels H.V., Beasley J.M., Sullivan C.V., Borski R.J., 2004. Induction of diploid gynogenesis in southern flounder (*Paralichthys lethostigma*) with homologous and heterologous sperm. Aquaculture 237, 499-516.

Morgan A.J., Murashige C.A., Woolridge J.A., Luckenbach W.O., Watanabe R.J., Borski J., Godwin H.V., Daniels H., 2006. Effective UV dose and pressure shock for induction of meiotic gynogenesis in southern flounder (*Paralichthys lethostigma*) using black sea bass (*Centropristis striata*) sperm. Aquaculture 259, 290-299.

Morse W.W., 1981. Reproduction of the Summer Flounder, *Paralichthys dentatus* (L.). Journal of Fish Biology 19, 189-203.

Morisawa M., Suzuki K., 1980. Osmolality and potassium ion: their roles in initiation of sperm motility in teleosts. Science 210, 1145-1147.

Munkittrick K.R., Moccia R.D., 1984. Advances in the cryopreservation of salmonid semen and suitability for a production-scale artificial fertilization program. Theriogenology 21, 645-660.

Mylonas C.C., Fostier A., Zanuy S., 2010. Broodstock management and hormonal manipulations of fish reproduction. General and Comparative Endocrinology. 165, 516-534.

Mylonas, CC; Zohar, Y., 2001. Endocrine regulation and artificial induction of oocyte maturation and spermiation in basses of the genus *Morone.* Aquaculture 202, 205-220.

NMFS. 2002. Fisheries, statistics and economic division. National Oceanic and Atmospheric Administration. Silver Spring, Maryland, USA.

NOAA. 2008. Summer flounder (*Paralichthys dentatus*). National Marine Fisheries Service, http://www.nmfs.noaa.gov/fishwatch/species/summer_flounder.htm.

Naylor R.L., Goldburg, R.J., Primavera J.H., Kautsky N., Beveridge M., Clay J., Folke C., Lubchenco J., Mooney, Harold T., Max, 2000. Effect of aquaculture on world fish supplies. Nature 405, 1017-1024.

Negatu Z., Hsiao S.M., Wallace R.A ., 1998. Effects of insulin-like growth factor-I on final oocyte maturation and steroid production in *Fundulus heteroclitus.* Fish Physiology and Biochemistry 19, 13-21.

Peatpisut T., Amrit B.N., 2010. Cryopreservation of sperm from natural and sex-reversed orange-spotted grouper (*Epinephelus coioides*). Aquaculture Research 42, 22-30.

Rana K.J., 1995. Cryopreservation of fish spermatozoa. In: Methods in Molecular Biology 38: Cryopreservation and freeze-drying protocols (ed. by J.G. Day and M.R. McLellan) p. 151-165. Totowa, NJ.

Rideout R.M., Litvak M.K., Trippel E.A., 2003. The development of a sperm cryopreservation protocol for winter flounder *Pseudopleuronectes americanus* (Walbaum): evaluation of cryoprotectants and diluents. Aquaculture Research 34, 653-659.

Rondán M., Hernandez M.D., Egea M.A., Garcia B., Jover M., Rueda F.M., 2004. Effects of fishmeal replacement with soybean meal as protein source, and protein replacement with carbohydratesas an alternative energy source on sharpsnout sea bream, *Diplodus puntazzo,* fatty acid profile. Aquaculture Research 35, 1220-1227.

Routray P., Dash S.N., Dash C., Swain P., Sarkar Sampad K., Sarangi N., 2008. Cryopreservation of silver barb *Puntius gonionotus* (Bleeker) spermatozoa: effect of extender composition, cryoprotective agents and freezing rate on their post-thawing fertilization ability. Aquaculture Research 39, 1597-1605.

Sarkar S.K., Saha A., Dasgupta S., Nandi S., Verma D.K., Routray P., Devaraj C., Mohanty J., Sarangi N., Eknath A.E., Ayyappan S., 2010. Photothermal manipulation of reproduction in Indian major carp: A step forward for off-season. breeding and seed production
Current Science 99, 960-964.

Scott A.P., Baynes. 1980. A review of the biology, handling and storage of salmonid spermatozoa. Journal of Fish Biology 17, 707-739

Schreck C.B., Contreras-Sanchez W., Fitzpatrick M.S., 2001. Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197, 3-24.

Schulz R.W., Miura T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiology and Biochemistry 26, 43–56.

Seikai, T., 2000. Flounder culture, Japanese. Pages 382-387 in R. R. Stickney, editor. Encyclopedia of Aquaculture, Wiley, Ney York, New York, USA.

Stacey N., Fraser E.J., Sorensen P., Van Der Kraak G., 2001. Milt production in goldfish: regulation by multiple social stimuli. Comparative Biochemistry and Physiology. 130, 467-476.

Stoss J., Donaldson E.M., 1983. Studies on cryopreservation of eggs from rainbow trout (*Salmo gairdneri*) and coho salrnon (*Oncorhynchus kkutch*). Aquaculture 31, 51-65.

Suquet M., Dreanno C., Fauvel C., Cosson J., Billard, R., 2000. Cryopreservation of sperm in marine fish. Aquaculture Research 31, 231-243.

Tabata K., Gorie S., Nakamura K., 1986. Induction of gynogenetic diploid in hirame *Paralichthys olivaceus*. Bulletin of the Japanese Society of Scientific Fisheries 52, 1901-1904.

Tidwell J.H., Coyle S.D., Bright L.A., Yasharian D., 2005. Evaluation of plant and animal source proteins for replacement of fish meal in practical diets for the large mouth bass *Micropterus salmoides*. Journal of the World Aquaculture Society 36, 454-463.

Trippel E.A., Neilson J.D., 1992. Fertility and sperm quality of virgin and repeat-spawning Atlantic cod (*Gadus morhua*) and associated hatching success. Canadian Journal of Fisheries and Aquatic Sciences 49, 2118-2127.

Veillette, P.A., M. Merino, N.D. Marcaccio, M.M. Garcia and J.L. Specker., 2007. Cortisol is necessary for seawater tolerance in larvae of a marine teleost the summer flounder. General and Comparative Endocrinology 151, 116-121.

Vizziano D., Fostier A., Loir M., Le Gac F., 2008. Testis development, its hormonal regulation and spermiation induction in teleost fish. Fish Spermatology 3, 103-139.

Wang Z., Crim L.W., 1997. Seasonal changes in the biochemistry of seminal plasma and sperm motility in the ocean pout *Macrozoarces americanus*. Fish Physiology and Biochemistry 16, 77-83.

Webber H.H., Riordan, P.F., 1975. Criteria for candidate species for aquaculture In: Proceedings of the Sixth Annual Meeting [of the] World Mariculture Society held at Seattle, Washington, January 27-31, 1975 in cooperation with [the] University of Washington, Seattle [and the] National Marine Fisheries Service. World Mariculture Society, Charleston, SC (USA) Publ. by: Louisiana State University. Division of Continuing Education; Baton Rouge, LA (USA). 389-406.; Annual Meeting., Proceedings of the World Mariculture Society.

Wolters W.R., Libey G.S., Chrisman C.L., 1982. Effect of triploidy on growth and gonad development of channel catfish. Transactions of the American Fisheries Society 111, 102-105.

Yamamoto E., 1999. Studies on sex-manipulation and production of cloned populations in hirame, *Paralichthys olivaceus*. Aquaculture 173:235-246.

Yang H., Tiersch T.R., 2009. Review: Current status of sperm cryopreservation in biomedical research fish models: Zebrafish, medaka, and Xiphophorus Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology 149, 224-232.

Watanabe W.O., Carroll P.M., 2001. Progress in controlled breeding of summer flounder, *Paralichthys dentatus*, and southern flounder, *Paralichthys lethostigma*. Journal of Applied Aquaculture 11, 89-111.

Zhang Y.Z., Zhang, S.C., Liu, X.Z., Xu, Y.Y., Wang, C.L., Sawant, M.S., Li, J., and Chen, S.L., 2003. Cryopreservation of flounder (*Paralichthys olivaceus*) sperm with a practical methodology. Theriogenology 60, 989-996.

CHAPTER I

CRYOPRESERVATION OF SUMMER FLOUNDER, (*PARALICTHYS DENTATUS*) SPERM

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Abstract

The summer flounder, *Paralichthys dentatus* L., is a high value species and considerable research has been conducted to determine practices conducive for its culture. As milt can be limited in this species, experiments were conducted to develop a practical sperm cryopreservation protocol for hatchery use. Two dilution ratios (1:2 and 1:4; sperm:extender), 2 diluents (saline and sucrose-based), 2 cryoprotectants (10% DMSO and 12% glycerol) and 3 freezing rates (-5, -10 and -15°C min^{-1}) were evaluated using differential staining to assess post-thaw sperm survival. Seven combinations of the factors examined reduced post-thaw viability by less than 30%. The average viability of sperm from fresh, pooled flounder milt (67.2 ± 2.9%) was not different from that of thawed milt diluted 1:4 with sucrose diluent (10% DMSO) frozen at -5°C min^{-1} (38.4 ± 7.7%) and fertilization and hatch success were not different in trials using fresh or thawed, cryopreserved sperm. From these experiments a practical sperm cryopresrvation method was developed, but further refinement of the freezing protocol is necessary to optimize results.

Introduction

Paralichthid flounder are widely cultured throughout the world, particularly in Asia, where Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel), represents one of the most important aquaculture industries (Bolasina, Tagawa, Yamashita & Tanaka 2006). In the U.S., considerable research has been conducted to determine practices suitable for the culture of summer flounder, *P. dentatus* L. (Bengtson 1999; Burke, Seikai, Tanaka & Tanaka 1999; Watanabe & Carroll 2001; Gaylord, Schwarz, Cool, Jahncke & Craig 2004). Summer flounder are a high-value species and fishing pressure has significantly reduced wild catches, such that demand often exceeds supply (NOAA 2008). Meeting the increased demand for flounder in U.S. and foreign markets will therefore require establishing and optimizing production through aquaculture, and limited commercial production has been underway in the US for over a decade (Bengtson 2000).

Milt production from Paralichthid flounder can be limited (< 0.5 ml) during manual spawning and methods must be developed to maximize production from male broodstock (Smigielski 1975; Berlinsky, King, Hodson & Sullivan 1997). In addition to hormonal therapies to increase sperm production, sperm cryopreservation is another tool that can be used to manage valuable broodstock (Chao & Liao 2001; Riley, Holladay, Chesney & Tiersch 2004; Horváth, Urbányi, Mims, Bean, Gomelsky & Tiersch 2006). Some of the benefits of cryopreservation include synchronizing gamete availability during hatchery spawning, preservation of genetically superior pedigree lines, simplified sperm transport between hatcheries, reduction in disease transfer among broodstock and

reduced maintenance costs of male broodstock (Suquet, Dreanno, Fauvel, Cosson & Billard 2000; Jenkins-Keeran, Schreuders, Edwards & Woods 2001; Cabrita, Sarasquete, Martinez-Páramo, Robles, Beirão, Pèrez-Cerezales & Herráez 2010).

Several factors have been shown to contribute to the relative success of sperm cryopreservation, including freezing and thawing rates, choice of cryoprotectants and diluents, dilution ratios, sperm volumes, freezing vessels and variation among individual males (Suquet *et al.* 2000). There have been successful efforts to develop cryopreservation methods for other Paralichthid species including Japanese and Brazilian flounder, *P. orbignyanus* (Valenciennes), (Zhang, Zhang, Liu, Xu, Wang, Sawant, Li & Chen 2003; Lanes, Okamoto, Cavalcanti, Collares, Campos, Deschamps, Robaldo, Marins & Sampaio 2008), but to date, no methods have been reported for summer flounder. The objective of the present study was to develop a practical method for cryogenic sperm storage from summer flounder combining parameters (diluent composition, cryoprotectants, dilution ratios, and freezing rates) previously used with other Paralichthid species.

Materials and Methods

Broodstock

Wild-caught (southern New England) and captive-bred summer flounder broodstock (> 7 years; females = 0.97-3.65 kg, males = 0.68-1.60 kg) were maintained at Great Bay Aquaculture LLC (GBA; Portsmouth, NH, USA) for at least four years prior to the start of the experiments. Each fish was implanted with a passive integrated

transponder (PIT) tag (Biomark Inc., Boise, ID, USA) for individual identification and held in 6,000 L fiberglass rectangular tanks incorporated in recirculating (28-30 g L^{-1} salinity) systems. The systems were equipped with biological and mechanical filtration, ultraviolet sterilization, foam fractionation, and photothermal control. Half-hour crepuscular periods were provided with 100 W incandescent bulbs to simulate dawn and dusk. Light intensity, measured with a light meter (Sper Scientific, Scottsdale, AZ, USA), ranged from 5 lux (dawn/dusk) to 30 lux (day) at the water surface during the light period. Water temperature and dissolved oxygen (DO) were measured daily (Oxyguard, Birkerod, Denmark) and unionized ammonia and nitrite were monitored weekly (HACH®; Loveland, CO, USA). Water quality within the culture tanks remained within ranges suitable for rearing this species (DO = 100% stauration; ammonia < 0.0008 mgL^{-1}, nitrate < 3.0 mgL^{-1}; Watanabe, Ellis & Ellis 1998). The fish were fed a commercial ration (9 mm pellet, 54% protein, 18% fat; Vitalis Cal, Skretting, New Brunswick, Canada) to apparent satiation 2-3 times per week. As summer flounder naturally spawn in the fall, photoperiod and temperature were maintained at 12L:12D and 19 ± 1°C until two months prior to desired spawning and then gradually adjusted to 8L:16D and 14°C, respectively (Watanabe *et al.* 1998; Bengtson 1999).

Milt collection

Before milt collection, flounder were anesthetized with 70 mg L^{-1} MS-222 (Tricane-S, Tricane Methanosulphonate, Western Chemical Inc., Scottsdale, AZ, USA) and the area around the urogenital pore was blotted dry with a paper towel. Milt was collected and volumes measured in 1 mL Tuberculin syringes (Beckton, Dickinson and

Co., Franklin Lakes, NJ, USA) and transferred into 2 mL microcentrifuge tubes. The milt was used immediately after verifying sperm motility, or held on ice < 1 h prior to use (Lanes *et al.* 2008).

Assessing sperm viability and motility

Sperm density was determined from 10 summer flounder males by diluting samples (1:1000) with 10% formalin and counting immotile cells using a Neubauer hemacytometer (West Germany) at 1000× with a compound microscope (Zeiss AxioCam MRm, Carl Zeiss Inc., Thornwood, NY, USA). All samples were counted in triplicate.

Sperm viability was determined using a LIVE/DEAD ® sperm viability kit (Molecular probes, Eugene, OR, USA) which differentially stains live and dead cells with SYBR 14 ® and propidium iodide dyes, respectively. The stained sperm (> 100) were viewed with a Zeiss Axiophot fluorescence microscope (Oberkochen, Germany) using green fluorescence protein and rhodamine filters. Sperm viability was calculated as the proportion of live cells relative to the total number of cells counted.

Before use, sperm (diluted 1:50; 1 µl milt : 49 µl seawater) were checked for motility with a compound microscope immediately after activation with ultraviolet-sterilized seawater (30 g L^{-1}). Motility was estimated with an arbitrary scale, ranging from 0-4, where 0 represents no motility; 1, 1- 25%; 2, 26-50%; 3, 51-75%; and 4, 76-100% motile sperm (Viveiros, Jatzkowski & Komen 2003). Only sperm with motility scores ≥ 3 were used for cryopreservation trials.

Viability analysis

To validate the Live/Dead ® sperm viability kit, a regression analysis was

conducted in JMP 8.0 (SAS Institute, Inc. Cary, NC, USA) between percent viable sperm and qualitative motility (n = 21; Figure 1).

Cryopreservation experiment 1

To identify the optimal parameters for cryopreservation, a multi factorial (2 diluents x 2 cryoprotectants x 2 dilution ratios x 3 freezing rates) ANOVA experimental design was conducted using sperm collected from four males and frozen using one of two diluents (sucrose: 110 mM sucrose, 100 mM $KHCO_3$, 10 mM Tris-Cl, pH 8.2, osmolality 335 mOsmol; and saline: 423 mM NaCl, 9 mM KCl, 9.25 mM $CaCl_2 \cdot 2H_2O$, 22.92 mM $MgCl_2 \cdot 6H_2O$, 25.5 mM $MgSO_4 \cdot 7H_2O$, 2.15 mM $NaHCO_3$, pH 8.2, osmolality 900mOsmol), two cryoprotectants (12% or 1.65 M glycerol; and 10% or 1.40 M DMSO) % cryoprotectant indicates final cryoprotectant concentration of the sample, two dilution ratios (1:2; 100 µl:100 µl and 1:4; 50 µl:150 µl), and three freezing rates (-5, -10, and -15°C min^{-1}). The diluents were mixed with cryoprotectants approximately 1 day prior to use, and were held at 4°C. All solutions and instruments used in the freezing procedure were chilled (4°C) prior to use. The milt was diluted in 2 mL cryo-vials (Nalgene Cryoware Low density polyethylene, Nalgene Co. Rochester, NY, USA). Vials were capped, thoroughly mixed, and held on ice until all replicates for one freezing rate were filled. No additional equilibration time was allotted beyond that required to fill the cryo-vials (2-3 min). Cryo-vials containing sperm samples were frozen using a Planar Biomed Model 10-16 programmable freezer (Planer, United Kingdom). Once samples reached -150°C, they were immediately plunged into liquid nitrogen and kept overnight. The samples were thawed in a 37°C water bath for 2 min, and held at room temperature

(20°C). The sperm were immediately examined using the LIVE/DEAD ® sperm viability kit, as described above. Each diluent, cryoprotectant, and dilution combination was frozen in triplicate at each of the 3 freezing rates. Because different pools of sperm were used for each freezing rate trial, thawed sperm viability was compared to that from the corresponding fresh sperm pool. A least squares analysis was performed to compare freezing parameters (diluent, cryoprotectant, dilution ratio and freezing rate) and results are presented as "mean decrease in viability."

Cryopreservation experiment 2

The results of the initial cryopreservation experiment were further analysed using the maximize desirability function in the prediction profiler (JMP 8.0). This function compared treatments and interactions and selected the combination of treatment levels that yielded the greatest post-thaw viability. From this analysis, the cryoprotectant (DMSO) and dilution (1:4) were selected for further analysis in a 2x3 factorial design and combined with both diluents, at each freezing rate. Sperm from four males were frozen with these parameters in triplicate, as described above.

Egg collection

During all procedures fish were anesthetized with 70 mg L^{-1} MS-222. Two weeks prior to anticipated spawning, ovarian development was visually assessed with the aid of a light table (Watanabe & Carroll 2001; Luckenbach, Godwin, Daniels & Borski 2002). Those fish containing vitellogenic stage oocytes were induced to spawn with daily injections of carp pituitary extract (CPE, 2 mg kg^{-1}; Stoller Fisheries, Spirit Lake, IA, USA; Smigielski 1975; Berlinsky *et al.* 1997). Females were checked for evidence of

ovulation daily by exerting gentle pressure on the dorsal surface overlying the ovaries. If ovulation did not occur, the degree of ovarian development was visually assessed and CPE was re-administered. This procedure was repeated daily (~3–4 days) until ovulation occurred.

Ovulated eggs were collected into a 500 mL polypropylene beaker and their total volume recorded. A subsample of eggs (n = 200) was examined to assess quality. High quality eggs from marine teleosts are generally clear, buoyant, spherical, and lack a perivitelline space prior to fertilization (McEvoy 1984; Kjørsvik, Mangor-Jensen & Holmefjord 1990). An estimate of the number of eggs exhibiting these characteristics was determined. If most of the eggs appeared to be of high quality, the batch was retained for fertilization.

Fertilization trial

Based on the results of the second cryopreservation experiment, fertility trials were conducted using sperm diluted 1:4 with sucrose diluent and DMSO solution, and frozen in 1 mL aliquots at -5°C min^{-1}. Eggs from three females were collected as above, divided into two 250 mL polypropylene beakers, and fertilized with either fresh milt (100 µL) or thawed, cryopreserved milt from four males (1-1.0 mL aliquot). Approximately 20-40 ml of filtered sea water (34 g/L) was added and the eggs and sperm were gently mixed for 2 min. Following fertilization the eggs were transferred to a calibrated separatory funnel containing 700–1000 mL seawater and statically incubated for 5-10 min to allow the buoyant (viable) and sinking (non viable) eggs to separate. The volumes of both groups of eggs were recorded and the number of eggs mL^{-1} was estimated (1,200

eggs mL^{-1}) based on previous findings (Berlinsky *et al.* 1997). The percentage of fertilized eggs was determined after 3-4 h (32-64 cell stage) by microscopic examination of approximately 200 eggs. The viable eggs were then incubated at GBA in 50 L incubators connected in a recirculating system (17°C, 35 g L^{-1} salinity, continuous illumination of approximately 200 lux). Each day, aeration was removed and water flow stopped for 10 min to allow non-viable eggs to sink. The volume of non-viable eggs was recorded daily, and the final buoyant volume was recorded at 72 h. Following examination with a dissecting microscope, pre-hatch success was determined as the ratio of developed embryos (beating hearts) to non-developed, in the final buoyant egg volume.

Statistical analysis

The effects of predictor parameters (diluent, cryoprotectant, dilution ratio, and freezing rate) on sperm viability and fertilization, with fresh and cryopreserved sperm, were analyzed by ANOVA and a full-factorial least squares analysis. When effects were significant, a Tukey's *a posteriori* multiple range test was used for pair-wise comparisons. Percent data were arcsine square-root transformed to improve the ANOVA assumption of normality. Linear regression analysis was conducted to determine the correlation between sperm motility. All statistics were performed using JMP 8.0. All data are presented as means ± SEM.

Results

Assessing sperm viability and motility

Flounder (n=10) had a milt volume of 1.9 ± 0.5 mL, a density of 12.4 ± 1.6 ($\times 10^9$) cells mL^{-1}, and a total cell count of 26.3 ± 6.6 ($\times 10^9$). The volume corrected for fish size was 1.5 ± 0.5 mL kg^{-1} BW. A positive relationship was verified between sperm motility and viability (n=21, Adjusted $R^2 = 0.81$, $p < 0.05$, Fig. 1).

Cryopreservation experiments

The results of cryopreservation experiment 1 are shown in Table 1.1 Seven combinations of the factors examined reduced post-thaw viability by less than 30%. Only the treatment "freezing rate" was statistically significant ($P < 0.0001$) and an interaction was detected between diluent and cryoprotectant ($P = 0.0008$). Overall the -5°C min^{-1} freezing rate (26.9% decrease in viability) outperformed -10 (50.0%) and -15°C min^{-1} (52.0%), and DMSO cryoprotectant (39.4%) outperformed glycerol (46.4%). The results of the subsequent cryopreservation trial, during which sperm were diluted 1:4 with either sucrose or saline diluents and frozen at different rates (Table 1.2), shows that sucrose outperformed saline. The average viability of sperm from fresh, pooled flounder milt (67.2 ± 2.9%) was not different from that of thawed sperm diluted 1:4 with sucrose diluent (10% DMSO) frozen at -5°C min^{-1} (38.4 ± 7.7%).

There were no statistical differences in fertilization success or pre-hatch viability between fresh and thawed sperm Table 1.3. The sperm to egg ratios, however, differed between fresh (34.7×10^3 sperm: egg) and cryopreserved (88.4×10^3 sperm: egg)

treatments.

Discussion

Sperm densities and milt volumes vary considerably among teleost species and are further influenced by season, social factors, stress, nutrition, and frequency of stripping (Büyükhatipoglu & Holtz 1984; Teletchea, Gardeur, Psenicka, Kaspar, Le Dore, Linhart & Fontaine 2009). Summer flounder are among several Pleuronectiform species that produce relatively low volumes of concentrated sperm (Lanes, Okamoto, Bianchini, Marins & Sampaio 2010). While cell motility is often used as an indicator of sperm quality, and the percentage of motile sperm has been positively correlated with fertilization in many species (Levanduski & Cloud 1988; Wang & Crim 1998; Fauvel, Suquet & Cosson 2010), in others, such as Atlantic cod, *Gadus morhua* L., (Trippel & Neilson 1992); salmonids (Scott & Baynes 1980), and rosy barb, *Barbus conchonius* (Hamiltion-Buchanan), (Amanze 1994) fertilization success using *in vitro* fertilization was not different using motile or immotile sperm. Differential staining of live and dead cells, based on their membrane integrity, has proven useful for rapid evaluation of preservation protocols prior to fertilization trials (DeGraaf & Berlinsky 2004; Lanes *et al.* 2008; Cabrita, Engrola, Conceição, Pousão-Ferreira & Dinis 2009), and was used in the present study. While high correlations between cell viability and motility have been reported for some species (e.g. cod, DeGraaf & Berlinsky 2004; Brazilian flounder, Lanes *et al.* 2008; summer flounder, present study), in other cases poor correlations were observed (Linhart, Rodina, Flajshans, Gela & Kocour 2005). This is likely due to the fact that live-staining populations also included cells with insufficient ATP reserves for

motility (Linhart & Billard 1994). Because of these discrepancies in viability assessment, the ultimate test for sperm quality is the ability to fertilize eggs (Bromage & Roberts 1995; Riley, Chesney & Tiersch 2008).

In the present study, fertilization and hatching were not different in trials using fresh or cryopreserved sperm. Adequate freezing success was achieved using a sucrose-based diluent with 10% DMSO, and results were similar to those reported in trials with the congeneric Brazilian flounder (Lanes *et al.* 2008). Although DMSO has proven widely successful for a number of marine teleosts, and has been called the "universal cryoprotectant" (Chao & Liao 2001), equal or greater success has been attained with other cryoprotectants. For instance, compared to DMSO, greater success was attained using glycerol, propylene glycol, and trehalose as sperm cryoprotectants for Japanese flounder, winter flounder, *Pseudopleuronectes americanus* (Walbaum), and orange-spotted grouper, *Epinephelus coioides* (Hamilton), respectively (Rideout, Litvak, & Trippel 2003; Zhang *et al.* 2003; Peatpisut & Amrit 2010).

Sperm cryopreservation has been applied to more than 200 marine and freshwater fish species and the degree to which specific variables influence post-thaw viability is highly species-dependent. For instance, in carp, *Cyprinus carpio* L. no differences in post-thaw motility were found using either sucrose or saline-based diluents (Horváth, Miskolczi & Urbányi 2003), but sucrose-based diluents were found to be superior to saline-based in spotted halibut, *Verasper variegatus* (Temminck & Schlegel), and turbot, *Scophthalmus maximus* L., (Chen, Ji, Yu, Tian & Sha 2004; Tian, Chen, Ji, Zhai, Sun, Chen & Su 2008). The cryopreservation trials reported here represent an important first

step towards effective male summer flounder broodstock management, and our protocol and results were similar to those of Lanes *et al.* (2008) for Brazilian flounder. Several cryopreservation parameter combinations gave satisfactory results in our initial trial, however, and greater amounts of cryopreserved sperm were necessary to attain the same fertility success as that of fresh sperm. Further refinement of the freezing protocol is necessary to optimize results, and will entail conducting sequential, stepwise comparisons of cryoprotectant concentrations, freezing and thawing rates, sperm diluents, and sperm to egg ratios (Suquet *et al.* 2000; Rideout *et al.* 2003).

In conclusion, several parameter combinations were successfully used for summer flounder sperm cryopreservation. Fertilization and hatch success were not different in trials using fresh or post-thawed cryopreserved sperm diluted 1:4 with a sucrose-based diluent and 10% DMSO. Further experiments may be necessary to optimize sperm cryopreservation techniques for this species

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References

Amanze D. (1994) Strategies for sperm-egg contact during external fertilization in teleosts. High performance fish: Proceedings of an International Fish Physiology Symposium at the University of British Columbia in Vancouver, Canada. July 16-21, pp. 176-181. Fish Physiology Association,Vancouver, BC, Canada.

Bengtson D.A. (1999) Aquaculture of summer flounder (*Paralichthys dentatus*): Status of knowledge, current research and future research priorities. *Aquaculture* 176, 39-49.

Bengtson D.A. (2000) Summer flounder culture. In: Encyclopedia of Aquaculture, John Wiley

Berlinsky D.L., King W.V., Hodson R.G. & Sullivan C.V. (1997) Hormone induced spawning of summer flounder, *Paralichthys dentatus*. *Journal of the World Aquaculture Society* 28, 79-86.

Bolasina S., Tagawa M., Yamashita Y. & Tanaka M. (2006) Effect of stocking density on growth, digestive enzyme activity and cortisol level in larvae and juveniles of Japanese flounder, *Paralichthys olivaceus*. *Aquaculture* 259, 432-443.

Bromage N.R. & Roberts R.J. (1995) Broodstock management and egg and larval quality. Blackwell Science, Cambridge, Massachusetts.

Burke J.S., Seikai T., Tanaka Y. & Tanaka M. (1999) Experimental intensive culture of summer flounder, *Paralichthys dentatus*. *Aquaculture* 176, 135-144.

Büyükhatipoglu S. & Holtz W. (1984) Sperm output in rainbow trout (*Salmo gairdneri*)—effect of age, timing and frequency of stripping and presence of females. *Aquaculture* 37, 63-71.

Cabrita E., Sarasquete C., Martinez-Páramo S., Robles V., Beirão J. & Pèrez-Cerezales & S., Herráez, M.P. (2010) Cryopreservation of fish sperm: applications and perspectives. *Journal of Applied Ichthyology* 26, 623-635.

Cabrita E., Engrola S., Conceição L.E.C., Pousão-Ferreira P. & Dinis M.T. (2009) Successful cryopreservation of sperm from sex-reversed dusky grouper, *Epinephelus marginatus*. *Aquaculture* 287, 152-157.

Chao N.H. & Liao I.C. (2001) Cryopreservation of finfish and shellfish gametes and embryos. *Aquaculture* 197, 161-189.

Chen S., Ji X., Yu G., Tian Y. & Sha Z. (2004) Cryopreservation of sperm from turbot (*Scophthalmus maximus*) and application to large-scale fertilization. *Aquaculture* 236, 547-556.

DeGraaf J.D., Berlinsky D.L. (2004) Cryogenic and refrigerated storage of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) spermatozoa. *Aquaculture* 234, 527-540.

Fauvel C., Suquet M. & Cosson J. (2010) Evaluation of fish sperm quality. *Journal of Applied Ichthyology* 26, 636-643.

Gaylord T.G., Schwarz M.H., Cool R.W., Jahncke M.L. & Craig S.R. (2004) Thermal optima for the culture of juvenile summer flounder, *Paralichthys dentatus*. *Journal of Applied Aquaculture* 14, 155-162.

Horváth Á., Miskolczi E. & Urbányi B. (2003) Cryopreservation of common carp sperm. *Aquatic Living Resources* 16, 457-460.

Horváth Á., Urbányi B., Mims S.D., Bean W.B., Gomelsky B., & Tiersch T.R. (2006). Improved cryopreservation of sperm of paddlefish (*Polyodon spathula*). *Journal of the World Aquaculture Society* 37, 356-362.

Jenkins-Keeran K., Schreuders P., Edwards K. & Woods III L.C. (2001) The effects of oxygen on the short-term storage of striped bass semen. *North American Journal of Aquaculture* 63, 238-241.

Kjørsvik E., Mangor-Jensen A. & Holmefjord I. (1990) Egg quality in fishes. In: Advances in Marine Biology, pp. 71–114. Academic Press Limited.

Lanes C.F.C., Okamoto M., Cavalcanti P.V., Collares T., Campos V.F., Deschamps J.C., Robaldo R.B., Marins L.F. & Sampaio L.A. (2008) Cryopreservation of Brazilian flounder (*Paralichthys orbignyanus*) sperm. *Aquaculture* 275, 361-365.

Lanes C.F.C., Okamoto M.H., Bianchini A., Marins L.F. & Sampaio L.A. (2010) Sperm quality of Brazilian flounder *Paralichthys orbignyanus* throughout the reproductive season. *Aquaculture Research* 41, e199-e207.

Levanduski M. & Cloud J. (1988) Rainbow trout (*Salmo gairderi*) semen: effect of non-motile sperm on fertility. *Aquaculture* 75, 171-179.

Linhart O. & Billard R. (1994) Spermiation and sperm quality of European catfish (*Silurus glanis* L.) after implantation of GnRH analogs and injection of carp pituitary extract. *Journal of Applied Ichthyology* 10, 182-188.

Linhart O., Rodina M., Flajshans M., Gela D. & Kocour M. (2005). Cropreservation of European catfish *Silurus glanis* sperm: sperm motility, viability, and hatching success of embryos. *Cryobiology* 51, 250-261.

Luckenbach J.A., Godwin J., Daniels H.V. & Borski R.J. (2002) Optimization of North American flounder culture: a controlled breeding scheme. *World Aquaculture* 33, 40-45.

McEvoy L.A. (1984) Ovulatory rhythms and over-ripening of eggs in cultivated turbot, *Scophthalmus maximus* L. *Journal of Fish Biology* 24, 437–448.

NOAA (2008) Summer flounder (*Paralichthys dentatus*). National Marine Fisheries Service, http://www.nmfs.noaa.gov/fishwatch/species/summer_flounder.htm.

Peatpisut T. & Amrit B.N. (2010) Cryopreservation of sperm from natural and sex-reversed orange-spotted grouper (*Epinephelus coioides*). *Aquaculture Research* 42, 22-30.

Rideout R.M., Litvak M.K. & Trippel E.A. (2003) The development of a sperm cryopreservation protocol for winter flounder *Pseudopleuronectes americanus* (Walbaum): evaluation of cryoprotectants and diluents. *Aquaculture Research* 34, 653-659.

Riley K.L., Chesney E.J. & Tiersch T.R. (2008) Field collection, handling, and refrigerated storage of sperm of red snapper and gray snapper. *North American Journal of Aquaculture* 70, 356-364.

Riley K.L., Holladay C.G., Chesney E.J. & Tiersch T.R. (2004) Cryopreservation of sperm of red snapper (*Lutjanus campechanus*). *Aquaculture* 238, 183-194.

Scott A.P. & Baynes S.M. (1980) A review of the biology, handling and storage of salmonid spermatozoa. *Journal of Fish Biology* 17, 707-739.

Smigielski A.S. (1975) Hormone-induced spawnings of the summer flounder and rearing of the larvae in the laboratory. *The Progressive Fish-Culturist* 37, 3-8.

Suquet M., Dreanno C., Fauvel C., Cosson J. & Billard R. (2000) Cryopreservation of sperm in marine fish. *Aquaculture Research* 31, 231-243.

Teletchea F., Gardeur J-N., Psenicka M., Kaspar V., Le Dore Y., Linhart O. & Fontaine P. (2009) Effects of four factors on the quality of male reproductive cycle in pikeperch *Sander lucioperca. Aquaculture* 291, 217-223.

Tian Y.S., Chen S.L., Ji X.S., Zhai J.M., Sun, L.J., Chen C. & Su P.Z. (2008) Cryopreservation of spotted halibut (*Verasper variegatus*) sperm. *Aquaculture* 284, 268-271.

Trippel E.A. & Neilson J.D. (1992) Fertility and sperm quality of virgin and repeat-spawning Atlantic cod (Gadus morhua) and associated hatching success. *Canadian Journal of Fisheries and Aquatic Sciences* 49, 2118-2127.

Viveiros A.T.M., Jatzkowski A. & Komen J. (2003) Effects of oxytocin on semen release response in African catfish (*Clarias gariepinus*). *Theriogenology* 59, 1905-1917.

Wang Z. & Crim L.W. (1997) Seasonal changes in the biochemistry of seminal plasma and sperm motility in the ocean pout *Macrozoarces americanus*. *Fish Physiology and Biochemistry* 16, 77-83.

Watanabe W.O. & Carroll P.M. (2001) Progress in controlled breeding of summer flounder, *Paralichthys dentatus*, and southern flounder, *Paralichthys lethostigma*. *Journal of Applied Aquaculture* 11, 89-111.

Watanabe W.O., Ellis E.P. & Ellis S.C. (1998) Progress in controlled maturation and spawning of summer flounder *Paralichthys dentatus* boodstock. *Journal of the World Aquaculture Society* 29, 393-404.

Zhang Y.Z., Zhang S.C., Liu X.Z., Xu Y.Y., Wang C.L., Sawant M.S., Li J. & Chen S.L. (2003) Cryopreservation of flounder (*Paralichthys olivaceus*) sperm with a practical methodology. *Theriogenology* 60, 989-996.

Tables and Figures

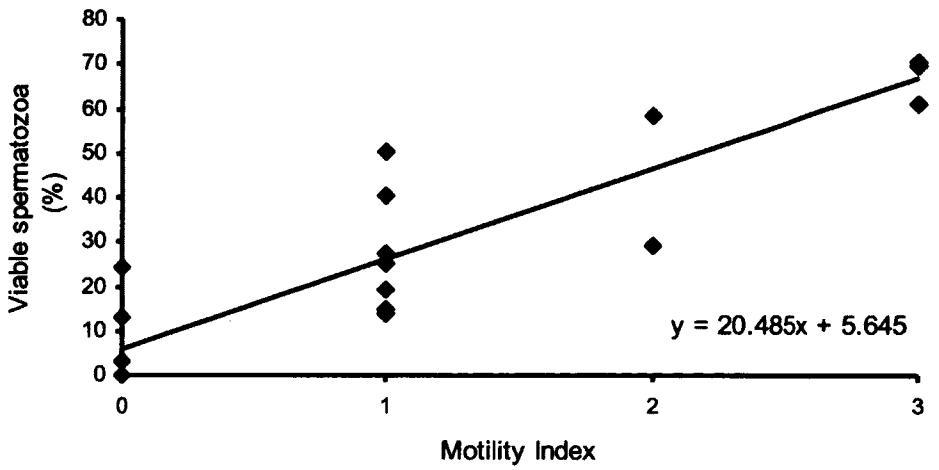

Figure 1.1 Correlation between summer flounder, *Paralichthys dentatus* L sperm motility and viability determined by differential fluorescent staining (n=21, Adjusted $R^2 = 0.81$, $p < 0.05$).

Table 1.1 Mean decrease in post-thaw viability for cryopreserved sperm from summer flounder, *Paralichthys dentatus* L. Values that share a letter are not significantly different (ANOVA, Tukey's Post Hoc, $p < 0.05$).

Dilution	Diluent	Cryoprotectant	Freezing Rate (°C min^{-1})	Decrease in viability post-thaw (%)
1:2	Saline	DMSO	-5	28.3 ± 11.7 abcd
1:2	Saline	DMSO	-10	58.4 ± 1.4 cd
1:2	Saline	DMSO	-15	46.2 ± 4.9 abcd
1:2	Saline	Glycerol	-5	18.9 ± 1.4 ab
1:2	Saline	Glycerol	-10	36.0 ± 14.4 abcd
1:2	Saline	Glycerol	-15	59.1 ± 3.7 cd
1:4	Saline	DMSO	-5	18.4 ± 8.5 a
1:4	Saline	DMSO	-10	58.6 ± 2.9 cd
1:4	Saline	DMSO	-15	58.7 ± 6.3 cd
1:4	Saline	Glycerol	-5	27.0 ± 8.6 abcd
1:4	Saline	Glycerol	-10	46.3 ± 5.8 abcd
1:4	Saline	Glycerol	-15	52.3 ± 7.8 abcd
1:2	Sucrose	DMSO	-5	21.6 ± 6.0 abc
1:2	Sucrose	DMSO	-10	46.9 ± 2.4 abcd
1:2	Sucrose	DMSO	-15	48.9 ± 2.9 abcd
1:2	Sucrose	Glycerol	-5	38.5 ± 3.9 abcd
1:2	Sucrose	Glycerol	-10	58.7 ± 1.4 cd
1:2	Sucrose	Glycerol	-15	57.6 ± 3.5 bcd
1:4	Sucrose	DMSO	-5	21.2 ± 8.3 abc
1:4	Sucrose	DMSO	-10	29.8 ± 13.9 abcd
1:4	Sucrose	DMSO	-15	36.3 ± 6.8 abcd
1:4	Sucrose	Glycerol	-5	40.9 ± 10.2 abcd
1:4	Sucrose	Glycerol	-10	64.9 ± 0.8 d
1:4	Sucrose	Glycerol	-15	56.4 ± 6.9 abcd

Table 1.2 Mean viability of fresh and post-thaw summer flounder, *Paralichthys dentatus* L., sperm determined by differential staining. All samples were frozen with extenders containing 10% DMSO at a 1:4 dilution. Values that share a letter are not significantly different (ANOVA, Tukey's Post Hoc Test, $p < 0.05$).

Diluent	Freezing Rate (°C min^{-1})	Viability (%)
Fresh	-	67.2 ± 2.9 a
Sucrose	-5	38.4 ± 7.7 ab
Sucrose	-10	35.0 ± 11.9 bc
Sucrose	-15	27.3 ± 1.3 bcd
Saline	-5	4.3± 4.3 d
Saline	-10	5.7 ± 4.1cd
Saline	-15	4.9 ± 4.9 cd

Table 1.3 Mean fertilization and pre-hatch development of summer flounder, *Paralichthys dentatus* L., eggs fertilized with fresh and cryopreserved sperm. Fertilization and prehatch results are presented as percentages of initial buoyant eggs.

Milt Condition	Initial Buoyant Eggs ($\times 10^3$)	Fertilization (%)	Pre-hatch (%)
fresh	40.4 ± 9.4	73.4 ± 5.5	55.73 ± 6.7
frozen	35.6 ± 2.8	62.8 ± 18.7	32.65 ± 15.9

CHAPTER II

SWIMMING CHARACTERISTICS AND FERTILIZING CAPACITY OF MEIOGYNOGENETIC (*PARALICTHYS DENTATUS*) SPERMATOZOA

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Abstract

Summer flounder exhibit sexually dimorphic growth rates, with females growing considerably faster and larger than males. In an effort to produce monosex (all female) populations, meiogynogenetic fish were produced and raised at male-determining temperatures. Upon attaining sexual maturity, spermatozoa characteristics from normal and meiogynogenetic (meiogyn) summer flounder were compared using computer-assisted sperm analysis. Sperm concentration was lower for meiogyn fish, but not when normalized for body weight, and swimming characteristics of each group were similar. In a fertilization trial using pooled eggs from 2 females, sperm from normal and meiogyn males had equal fertilization success, but fewer embryos produced from meiogyn sperm survived through development. Twenty-four hour survival of hatched larvae was equal from both groups (> 96%). Sperm from meiogyn males were used to fertilize eggs from seven domesticated female broodstock during commercial production. Mean fertilization and hatch were 56.0 ± 6.8% and 32.7 ± 8.9%, respectively, resulting in the production of 304,450 larvae.

Keywords: Summer flounder; *Paralichthys dentatus*; CASA; meiogynogenetic

Introduction

Due to high consumer demand, Paralichthid flounder are widely cultured throughout the world, particularly in Asia, where Japanese flounder, *Paralichthys olivaceus*, culture represents one of the most significant marine aquaculture industries (Bolasina et al. 2006). In the U.S., considerable research has been conducted to determine practices conducive for the culture of summer flounder, *P. dentatus*. Studies to date have focused on methods to improve spawning, larviculture, juvenile rearing, and disease prevention and management (Bengtson 1999; Burke et al., 1999; Watanabe and Carroll 2001; Gaylord et al. 2004; Veillette et al. 2007) and limited, commercial production has been underway in the USA for over a decade, primarily in recirculating systems (Bengtson and Nardi 2000).

Growth rate remains the dominant factor controlling profitability of land-based culture of this species, and the costs associated with juvenile growth to market size must be reduced to gain competitiveness on the global market (King et al. 2001). Summer flounder, like other Paralichthid species, exhibit sexually dimorphic growth rates, with females growing considerably faster and larger than males (Morse 1981; King et al. 2001). A tremendous increase in growth performance can therefore be realized through the production of all-female populations of fingerlings. In one study, (King et al. 2001) found that during routine production, female summer flounder grew 1.4 times larger than males at 15 months post hatch, and were projected to be twice as large by harvest at 23 months. Monosex populations of Japanese flounder have been produced commercially in Japan and Korea since 1990 and 1995, respectively (Yamamoto 1999; Seikai 2000).

Of Paralichthid species, the specific sex-determining mechanisms have only been definitively elucidated for Japanese flounder. Investigations have shown that Japanese flounder females, like many teleosts with an "XX/XY" sex chromosome system, are homogametic (XX) and males heterogametic (XY) (Tabata 1991; Yamamoto 1999). During the sex-determining period of development, however, homogametic individuals can be phenotypically sex reversed by exposure to high water temperatures or exogenous steroids (Kitano et al. 1999; Kitano 2002). These XX-males can then be reared to maturity, distinguished from XY males in the population by progeny testing, and crossed with normal, XX females to produce monosex populations (Hattori et al. 2007). To avoid the time and expense associated with progeny testing, populations of fish possessing only XX, maternal genotypes have been produced by diploid gynogenesis and then sex-reversed (Tabata et al. 1986; Tabata 1991; Hulata 2001; Devlin and Nagahama 2002).

Diploid gynogenesis has also been accomplished for summer (Colburn et al. 2009) and southern flounder, *P. lethostigma* (Luckenbach et al. 2004; Morgan et al. 2006) and meiogyn fish from both species have been raised to maturity under male-determining environmental conditions. The objectives of the present study were to compare spermatozoa characteristics from sex-reversed, meiogyn and normal male summer flounder using computer-assisted sperm analysis (CASA). Fertilization capacity from both populations was then compared, and sperm from sex-reversed, meiogyn fish was used for *in vitro* fertilization during commercial production.

Materials and Methods

Brood stock

Wild-caught and captive-bred (normal) summer flounder broodstock (> 7 years; females = 0.97-3.65 kg, males = 0.68-1.60 kg) were maintained at Great Bay Aquaculture LLC (GBA; Portsmouth, NH, USA) for at least four years prior to the start of the experiments. The fish were implanted with a passive integrated transponder (PIT) tag (Biomark Inc., Boise, ID, USA) for individual identification and held in 6,000 - L fiberglass rectangular tanks incorporated in recirculating (28-30 ppt salinity) systems. The systems were equipped with biological and mechanical filtration, ultraviolet sterilization, foam fractionation, and photothermal control. Half-hour crepuscular periods were provided with 100 W incandescent bulbs to simulate dawn and dusk. Light intensity, measured with a light meter (Sper Scientific, Scottsdale, AZ, USA), ranged from 5 - lx (dawn/dusk) to 30 - lx (day) at the water surface during light period. Water temperature and dissolved oxygen were measured daily (Oxyguard, Birkerod, Denmark) and total ammonia nitrogen and nitrite were monitored weekly (HACH®; Loveland, CO USA). Water quality within the culture tanks remained within ranges suitable for rearing this species (Watanabe et al. 1998). The fish were fed a commercial ration (9 - mm pellet, 54% protein, 18% fat; Vitalis Cal, Skretting, Trouw, Spain) to apparent satiation 2-3 times per week. As summer flounder naturally spawn in the fall, photoperiod and temperature were maintained at 12L: 12D and 19 ± 1 C until two months prior to desired spawning and then gradually adjusted to to 8L: 16D and 14 C, respectively (Watanabe et al. 1998; Bengtson 1999).

Meiogynogenetic fish (0.24-0.77 kg) were produced as reported previously (Colburn et al. 2009). Briefly, summer flounder eggs were activated with ultraviolet-irradiated black sea bass, *Centropristis striata* sperm, and a 6 - min pressure shock (8,500 psi) was applied 2 min post-fertilization. The eggs and larvae were incubated (16-17 C and 35 ppt) and reared (16-18 C and 27-31 ppt) under standard hatchery conditions and fed enriched rotifers followed by *Artemia*. Upon metamorphosis (41 days-post hatch; DPH), the juveniles (n = 900) were transferred to 235 - L cylindrical tanks that were incorporated into recirculating systems and subjected to male-determining temperatures ($\geq$ 21 C). At 376 DPH, 153 of the remaining fish were transferred to the broodstock systems described above, and held for approximately 3 years prior to the start of the experiments.

Sperm Collection

Before sperm collection, flounder were anesthetized with 70 mg/L tricaine methanesulphonate (MS-222; Western Chemical Inc., Scottsdale, AZ, USA) and the area around the urogenital pore was blotted dry with a paper towel. Sperm were collected and volumes measured in 1- mL Tuberculin syringes (Beckton, Dickinson and Co. Franklin Lakes, NJ, USA) and transferred into 2 - mL microcentrifuge tubes. The sperm were used immediately after verifying motility, or held on ice < 1- h prior to use (Lanes et al. 2008).

Assessing Sperm Motility and Viability

Sperm cell density were determined from 10 normal and 18 meiogyn fish by diluting samples (1:1000) with 10% formalin and counting immotile cells using a

Neubauer hemocytometer (West Germany) at 1000 × with a compound microscope (Zeiss AxioCam MRm, Carl Zeiss Inc., Thornwood, NY, USA). All samples were counted in triplicate.

Before use, sperm was checked for motility with a compound microscope immediately following activation with ultraviolet-sterilized seawater (UVSW; 30 ppt). Dilution was equal to 1:50 (1 µL sperm and 49 µL seawater). Motility was estimated with an arbitrary scale, ranging from 0-4, where 0 represents no motility; 1, 1- 25%; 2, 26-50%; 3, 51-75%; and 4, 76-100% motile spermatozoa (Viveros et al. 2003). Only sperm with a motility scores ≥ 3 were used for fertilization trials and commercial production.

CASA

Sperm were collected from 8 normal and 9 meiogyn fish, analyzed for cell density as above, and stored undiluted in microcentrifuge tubes on ice < 1 h prior to analysis. The CEROS Analyzer used a multi-parameter approach to simultaneously assess many spermatozoa characteristics, including path velocity (VAP), track speed (VCL), progressive velocity (VSL), linearity (LIN), duration (DUR), and motility (MOT) Table 2.1. Video recordings of spermatozoa were analyzed every 30 sec for a 10 sec duration, until sperm motility ceased.

Egg Collection

During all procedures fish were anesthetized with 70 mg/L MS-222. Two weeks prior to anticipated spawning, ovarian development was visually assessed with the aid of a light table (Watanabe and Carroll 2001; Luckenbach et al. 2002). Those fish containing vitellogenic stage oocytes were induced to spawn with daily injections of carp pituitary

extract (CPE, 2 mg/kg; Stoller Fisheries, Spirit Lake, IA, USA; Smigielski 1975; Berlinsky et al. 1997). Females were checked for evidence of ovulation daily by exerting gentle pressure on the dorsal surface overlying the ovaries. If ovulation did not occur, the degree of ovarian development was visually assessed and CPE was re-administered. This procedure was repeated daily (~3–4 d) until ovulation occurred.

Ovulated eggs were collected into a 500 - mL polypropylene beaker and their total volume recorded. A subsample of eggs (n = 200) was examined to assess quality. High quality eggs from marine teleosts are generally clear, buoyant, spherical, and lack a perivitelline space prior to fertilization (Kjorsvik et al. 1990; Larsson et al. 1997). An estimate of the number of eggs exhibiting these characteristics was determined. If most of the eggs appeared to be of high quality, milt was added.

Fertilization Trial

To compare the fertility between normal (n=3) and meiogyn (n=4) males, milt was collected, and density enumerated, as described above, and used to fertilize the pooled eggs from 2 females. Three, 20-mL egg aliquots were fertilized with 100 µL of each sperm pool in 100-mL beakers, activated with approximately 25 mL filtered sea water (34 ppt), and gently mixed for 2 min. The eggs were transferred to a calibrated separatory funnel containing 700-800 mL seawater and statically incubated for 15 min to allow the buoyant (viable) and sinking (nonviable) eggs to separate. The volumes of both groups of eggs were recorded. The number of eggs/mL was estimated based on previous verification (G. Nardi, unpublished data) and the percentage of fertilized eggs was

determined after 2 - h (4-8 cell stage) by microscopic examination of approximately 200 eggs.

The buoyant eggs were transferred to six 50 - L incubators that were incorporated into a recirculating system and held at 17 C, 35 ppt with supplemental aeration. Nonviable embryos were collected from the bottoms of the incubators daily and enumerated volumetrically. Pre-hatch embryos (visible movement, beating hearts) were enumerated volumetrically after approximately 70 - h of incubation and returned to the incubators. Upon hatch, 20 larvae from each incubator were transferred to 1- L beakers containing 35 ppt seawater, 17° C with supplemental aeration and viability was assessed after 24 hours.

Commercial Production

Commercial-scale summer flounder production was performed at GBA using the eggs from 7 females, following the spawning protocols described above. All eggs were fertilized with milt from 6-8 meiogyn males at a ratio of 50 μL milt: 10 mL eggs. Following buoyancy separation, the viable eggs were pooled into four 100 - L incubators and incubated until hatching. A subset of approximately 200 eggs was retained to determine fertilization success. Upon hatching, the flow and aeration were stopped for 10 - min to allow the yolk sac fry to float to the surface. The larvae were transferred to 15 - L tanks containing aerated seawater and counted volumetrically by mixing the larvae and withdrawing samples with a 10 - mL serological pipette. This procedure was repeated six times, the larvae count averaged, and multiplied by the tank volume. Hatch success was determined as the ratio of hatched larvae to the number of buoyant eggs initially stocked in the incubator.

Statistical Analysis

Spermatozoa characteristics, fertilization, pre-hatch viability, hatching success, and larval survival were analyzed by ANOVA. Percent data were square-root arcsine transformed to improve the ANOVA assumption of normality. When effects were significant ($P < 0.05$), a Tukey's *a posteriori* multiple range test was used for pair-wise comparisons. All statistics were performed using JMP 8.0 & 9.0 software (SAS Institute, Inc., Cary, NC, USA).

Results

Assessing Sperm Viability and Motility

Body weight, sperm volume, and cell concentration were lower for meiogyn compared to normal fish, but sperm concentration did not differ between the groups when normalized for fish weight (Table 2.2).

CASA

For the most commonly reported spermatozoa characteristics (VAP, VCL, VSL, LIN, DUR, MOT), there were no significant differences found between normal and meiogyn summer flounder. These results correspond favorably with those from other species (Table 2.3).

Fertilization Trial

The results of the fertilization trial comparing sperm from normal and meiogyn fish are shown in Table 2.4. Egg buoyancy, fertilization success, and 24-h larval survival

were not different between the two groups, but development through hatching was lower using sperm from meiogyn (36.3%) compared to normal (46.6%) fish (Table 2.4.)

Commercial Production

In the commercial production at GBA, seven females ovulated a total of 11 times over a 3 d period. Eggs fertilized with meiogynogenetic male sperm (~19 x 10^3 spermatozoa: egg) had a mean fertilization and hatch of 55.0 ± 6.8% and 32.7 ± 8.9%, respectively (Table 5). A total of 304,450 larvae were produced.

Discussion

Sperm quality has been shown to be affected by a number of factors including broodstock nutrition and husbandry and post-harvest handling (Bobe and Labbé 2010). While several criteria have been used to assess sperm quality, none have been sufficiently integrative to fully predict the ability of spermatozoa to fertilize ova (Fauvel et al. 2010). Spermatozoa swimming ability of many animals, particularly mammals, has been assessed using CASA, and it is well suited for use with fish, since their spermatozoa motility is so short-lived (Fauvel et al. 2010). CASA has been used to determine environmental effects and potential toxicants on spermatozoa motility in many fish species, and also for evaluating sperm diluents prior to cryopreservation (Cosson 2008; Cosson et al. 2010). Despite CASA's usefulness, for these and other applications, sperm motility is not a definitive predictor of fertilizing potential, as in some species such as Atlantic cod (*Gadus morhua*) (Trippel and Neilson 1992), salmonids (Scott and Baynes 1980), and walleye (*Sander vitreus*) (Casselman et al. 2006), fertilization success was not

correlated with sperm motility. Further, motility and swimming parameters are not accurate indicators of genetic problems that can affect embryonic and larval development.

In the present study, no differences were detected by CASA in spermatozoa swimming parameters between normal and meiogyn fish, but the meiogyn fish were considerably smaller than normal 4-year old male broodstock fish previously raised at GBA, and in the fertilization trials, survival through hatching was lower compared to normal fish. Relatively low hatching (14-50%) was also observed in commercial production trials using meiogyn sperm. Decreased growth and fertility, due to homozygosity of deleterious alleles (Leary et al. 1985; Ihssen et al. 1990), have been reported in several meiogyn fish species, including the honmoroko, *Gnathopogon caerulescens* (Fujioka 1998), coho salmon, *Oncorhynchus kisutch* (Piferrer et al. 1994) rainbow trout, *Oncorhynchus mykiss* (Feist et al. 1995) and common carp, *Cyprinus carpio* (Komen et al. 1992). For instance, Feist et al. (1995) reported that survival of offspring produced from sex reversed meiogyn rainbow trout ranged from 0 to 62% of that from normal (control) fish. In other species (e.g. European sea bass, *Dicentrarchus labrax*, Felip et al. 2002, Franceson et al. 2005; Thai walking catfish, *Clarias macrocephalus* Na-Nakorn 1995) similar growth and fertility between meiogyn fish and controls were reported, and very high fertility was attained using cryopreserved sperm from sex reversed meiogyn Japanese flounder (Tabata and Mizuta 1997). Genetic selection during domestication has been shown to dramatically reduce sperm volume (Zohar 1996), and quality (Agnése et al. 1995) in commercially important species. Growth and fertility impairment in meiogyn

fish may be species-specific and/or related to the degree of selective pressure to which the broodstock had been subjected.

Fertilization success in fishes is often variable when using manual spawning and *in vitro* fertilization, and in the present study ranged from 15-90% during commercial application. This variation likely reflects differences in egg quality, which may be due to post-ovulatory aging (Bobe and Labbé 2010), but sperm quality and density cannot be excluded as contributing factors. Spermatozoa density differed between normal and meiogyn males used in the fertilization trial, but fertilization success did not differ despite using equal sperm volumes. This information, and the high fertilization often achieved during the commercial production study, suggests that adequate spermatozoa were available for *in vitro* fertilization. Although the optimal spermatozoa: egg ratio has not been determined for this species, ratios similar to that used in the present study (19, 000:1) were found to be adequate in other fish species such as walleye *Sander vitreus*, 25,000:1 (Rinchard et al. 2005); turbot *Scophthalmus maximus*, 9,000:1 (Chereguini et al. 1999) and Atlantic halibut *Hippoglossus hippoglossus*, 10,000:1 (Vermeirssen et al. 2000).

Sperm density and volume vary considerably among teleost species and are further influenced by season, social factors, stress, nutrition, and frequency of stripping (Büyükhatipoglu and Holtz 1984; Teletchea et al. 2009). Summer flounder are among several Pleuronectiform species that produce relatively low volumes of concentrated sperm (Lanes et al. 2010). The volumes of expressible sperm from meiogyn fish were lower than those reported for many other marine species (Suquet et al. 1994; Mylonas et

al. 2003), but expressible volumes less than 1 mL were also reported for turbot, *Psetta maxima* (Suquet et al. 1994), yellowtail flounder, *Limanda ferruginea* (Clearwater and Crim 1998), and Brazilian flounder, *Paralichthys orbignyanus* (Lanes et al. 2010). The relatively small size of the meiogyn fish may have limited sperm production, as in other species, sperm production has been shown to increase in older, larger individuals (Büyükhatipoglu and Holtz 1984; Billard 1986). The relatively low sperm production necessitated the use of several meiogyn males during commercial production, but the contribution of individual males to fertilization success is unknown. In other species such as Atlantic halibut, studies have confirmed that sperm competition is significant during *in vitro* fertilization, resulting in vastly skewed fertilization success among individuals (Ottesen et al. 2009). Additional studies are necessary to determine the optimal spermatozoa: ova ratio, and degree of sperm competition in summer flounder, to improve reproductive efficiency in this species.

In conclusion, spermatozoa from meiogyn summer flounder were similar to those produced from normal males when analyzed by CASA, but resulted in lower viability of developing embryos. Further experiments are necessary to optimize the spermatozoa: ova ratio in this species and resolve fertility differences between normal and meiogyn individuals. Sex-reversed meiogyn males were used in commercial-scale production and efforts are currently underway to evaluate the effects of temperature on sex differentiation of offspring from these individuals

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References

Agnése, J.F., Z.J. Otémé, and S. Gilles. 1995. Effects of domestication on genetic variability, fertility, survival and growth rate in a tropical siluriform: Hetrobranchus longifilis Valenciennes 1840. Aquaculture 131:197-204.

Bengtson, D.A. 1999. Aquaculture of summer flounder (Paralichthys dentatus): Status of knowledge, current research and future research priorities. Aquaculture 176:39-49.

Bengtson, D.A. and G. C. Nardi 2000. Summer flounder culture. Pages 907-913 *in* R. R. Stickney, editor. Encyclopedia of Aquaculture, Wiley, New York, New York, USA.

Berlinsky, D. L., W. V. King, R.G. Hodson, and C.V. Sullivan 1997. Hormone induced spawning of summer flounder, Paralichthys dentatus. Journal of the World Aquaculture Society 28:79-86.

Billard, R. 1986. Spermatogenesis and spermatology of some teleost fish species. Reproduction, Nutrition and Development 26:877-920.

Bobe, J. and C. Labbé 2010. Egg and sperm quality in fish. General and Comparative Endocrinology 165:535-548.

Bolasina, S., M. Tagawa, Y. Yamashita, and M. Tanaka 2006. Effect of stocking density on growth, digestive enzyme activity and cortisol level in larvae and juveniles of Japanese flounder, Paralichthys olivaceus. Aquaculture 259:432-443.

Burke, J. S., T. Seikai, Y. Tanaka, and M. Tanaka 1999. Experimental intensive culture of summer flounder, Paralichthys dentatus. Aquaculture 176:135-144.

Büyükhatipoglu, S. and W. Holtz 1984. Sperm output in rainbow trout (Salmo gairdneri) - effect of age, timing and frequency of stripping and presence of females. Aquaculture 37:63-71.

Casselman, S.J., A.I. Schultz-Hostedde, and R. Montgomerie 2006. Sperm quality influences fertilization success in walleye (Sander vitreus). Canadian Journal of Fisheries and Aquatic Sciences 63:2119-2125.

Chereguini, O., I. García de la Banda, I. Rasines and A. Fernandez 1999. Artificial fertilization in turbot, Scophthalmus maximus (L.): different methods and determination of the optimal sperm-egg ratio. Aquaculture Research 30:319-324.

Clearwater, S.J. and L.W. Crim 1998. Gonadotropin releasing hormone-analogue treatment increases sperm motility, seminal plasma pH and sperm production in yellowtail flounder Pleuronectes ferrugineus. Fish Physiology and Biochemistry 19:349-357.

Colburn, H. R., G. C. Nardi, R. J. Borski, and D.L. Berlinsky 2009. Induced meiotic gynogenesis and sex differentiation in summer flounder (Paralichthys dentatus). Aquaculture 289:175-180.

Cosson, J.J. 2008. Methods to analyze the movements of fish spermatozoa and their flagella. Pages 63-102 in Alavi, S.A., J.J. Cosson, K. Coward, and G. Raffiee, editors. Fish Spermatology. Alpha Science International, Ltd., Oxford, U.K.

Cosson, J.J., A.L. Groison, C. Fauvel, and M. Suquet 2010. Description of hake Merlucius merlucius spermatozoa: Flagellar wave characteristics and motility parameters in various situations. Journal of Applied Ichthyology 26:644-652.

Devlin, R.H. and Y. Nagahama 2002. Sex Determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. Aquaculture 208:191-364.

Fauvel, C., M. Suquet and J. Cosson 2010. Evaluation of fish sperm quality. Journal of Applied Ichthyology 26: 636-643.

Feist G., Y Choo-Guan., M.S. Fitzpatrick, and C.B. Schreck 1995. The production of functional sex-reversed male rainbow trout with 17 alpha-methyltestosterone and 11 beta-hydroxyandrostenedione. Aquaculture 131:145-152.

Felip, A., F. Piferrer, M. Carrillo, and S. Zanuy 2002. Growth, gonadal development and sex ratios of meiogynogenetic diploid sea bass. Journal of Fish Biology 61:347-359.

Francescon, A., A. Barbaro, D. Bertotto, A. Libertini, F. Cepollaro, J. Richard, P. Belvedere, and L. Colombo 2005. Assessment of homozygosity and fertility in meiotic gynogens of the European sea bass (Dicentrarchus labrax L.). Aquaculture 243:93-102.

Fujioka, Y. 1998. Survival, growth and sex ratios of gynogenetic diploid honmoroko. Journal of Fish Biology 52: 430-442.

Gaylord, T.G., M.H. Schwarz, R.W. Cool, M.L. Jahncke, and S.R. Craig 2004. Thermal optima for the culture of juvenile summer flounder, Paralichthys dentatus. Journal of Applied Aquaculture 14:155-162.

Groison, A.L., C. Fauvel, M. Susquet, O.S. Kjesbu, L.R. Le Coz, I. Mayer, and J. Cosson 2010. Some characteristics of sperm motility in European hake (Merluccius merluccius, L., 1758). Journal of Applied Ichthyology 26:682-689.

Hattori, R.S., R.J. Gould, T. Fujioka, T. Saito J. Kurita, C.A. Strussmann, M. Yokota, and S. Watanabe 2007. Temperature-dependent sex determination in Hd-Rr Medaka Oryzias latipes: Gender sensitivity, thermal threshold, critical period, and *Dmrt1* expression profile. Sexual Development 1:138-146.

Hulata, G. 2001. Genetic manipulations in aquaculture: A review of stock improvement by classical and modern technologies. Genetica 111:155-173.

Ihssen, P.E., L.R. McKay, I. McMillian, and R.B. Phillips 1990. Ploidy manipulation and gynogenesis in fishes: cytogenetic and fisheries applications. Transactions of the American Fisheries Society 119:698-717.

King N.J., Nardi G.C., Jones C.J. 2001. Sex linked growth divergence of summerflounder from a commercial farm: are males worth the effort? J. Appl. Aquac., 11, 77–88

Kitano, T. 2002. Studies on P450 Aromatase (P450arom) Gene expression in sex differentiation in Japanese flounder (Paralichthys olivaceus). Report of Kumamoto Prefectural Fisheries Research Center 5:7-24.

Kitano, T., K. Takamune, T. Kobayashi, Y. Nagahama, and S.I. Abe 1999. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (Paralichthys olivaceus). Journal of Molecular Endocrinology 23: 167-176.

Kjorsvik, E., A. Mangor-Jensen, and I. Holmefjord 1990. Egg quality in fishes. Advances in Marine Biology. Academic Press Limited, Oxford, U.K.

Komen, J., G.F. Wiegertjes, V.J.T. van Ginneken, E.H. Eding, and C.J.J Richter 1992. Gynogenesis in common carp (Cyprinus carpio L.). III. The effects of inbreeding on gonadal development of heterozygous and homozygous gynogenetic offspring. Aquaculture 104:51-66.

Krol, J., R.K. Kowalski, P. Hliwa, G.J. Dietrich, R. Stabinskiand, and A. Ciereszko 2009. The effects of commercial preparations containing two different GnRH analogues and dopamine antagonists on spermiation and sperm characterization in the European smelt Osmerus eperlanus (L). Aquaculture 286: 328-331.

Lanes, C.F.C., M. Okamoto, P.V. Cavalcanti, T. Collares, V.F. Campos, J.C. Deschamps, R.B. Robaldo, L.F. Marins, and L.A. Sampaio 2008. Cryopreservation of Brazilian flounder (Paralichthys orbignyanus) sperm. Aquaculture 275: 361-365.

Lanes, C.F.C., M.H. Okamoto, A. Bianchini, L.F. Marins, and L.A. Sampaio 2010. Sperm quality of Brazilian flounder Paralichthys orbignyanus throughout the reproductive season. Aquaculture Research 41:199-207.

Larsson, D.G.J., C.C. Mylonas, Y. Zohar, and L.W. Crim 1997. Gonadotropin-releasing hormone analogue (GnRHa) induces multiple ovulations of high-quality eggs in a cold water, batch-spawning teleost, the yellowtail flounder (Pleuronectes ferrugineus). Canadian Journal of Fisheries and Aquatic Sciences 54:1957-1964.

Leary, R.F., F.W. Allendorf, K.L. Kundsen, and G.H. Thorgaard 1985. Heterozygosity and developmental stability in gynogenetic diploid and triploid rainbow trout. Heredity 54: 219-225.

Liu, Q.H., J. Li, Z.Z. Xiao, F.H. Ding, D.D. Yu, and X.Z. Xu 2007. Use of computer-assisted sperm analysis (CASA) to evaluate the quality of cryopreserved sperm in red seabream (Pagrus major). Aquaculture 263:20-25.

Luckenbach, J.A., J. Godwin, H.V. Daniels, and R.V. Borski 2002. Optimization of North American flounder culture: a controlled breeding scheme. World Aquaculture 33: 40-45.

Luckenbach, J.A., J. Godwin, H.V. Daniels, J.M. Beasley, C.V. Sullivan, and R.V. Borski 2004. Induction of diploid gynogenesis in southern flounder (Paralichthys lethostigma) with homologous and heterologous sperm. Aquaculture 237:499-516.

Morgan, A.J., R. Murashige, C.A. Woolridge, J.A. Luckenbach, W.O. Watanabe, R.J. Borski, J. Godwin, and H.V. Daniels 2006. Effective UV dose and pressure shock for induction of meiotic gynogenesis in southern flounder (Paralichthys lethostigma) using black sea bass (Centropristis striata) sperm. Aquaculture 259:290-299.

Morse, W.W. 1981. Reproduction of the summer flounder, Paralichthys dentatus (L.). Journal of Fish Biology 19:189-203.

Mylonas, C.C., M. Papadaki, and P. Divanach 2003. Seasonal changes in sperm production and quality in the red porgy Pagrus pagrus (L.). Aquaculture Research 34:1161-1170.

Na-Nakorn, U. 1995. Comparison of cold and heat shocks to induce diploid gynogenesis in Thai walking catfish (Clarias macrocephalus) and performances of gynogens. Aquatic Living Resources 8:333-341.

Ottesen, O.H., I. Babiak, and G. Dahle 2009. Sperm competition and fertilization success of Atlantic halibut (Hippoglossus hippoglossus L.). Aquaculture 286:240-245.

Scott, A.P. and S.M. Baynes 1980. A review of the biology, handling and storage of salmonid spermatozoa. Journal of Fish Biology 17:707-739.

Seikai, T. 2000. Flounder culture, Japanese. Pages 382-387 *in* R. R. Stickney, editor. Encyclopedia of Aquaculture, Wiley, Ney York, New York, USA.

Smigielski, A.S. 1975. Hormone-induced spawnings of the summer flounder and rearing of the larvae in the laboratory. The Progressive Fish-Culturist 37:3-8.

Suquet, M., R. Billard, J. Cosson, G. Dorange, L. Chauvaud, C. Mugnier, and C. Fauvel 1994. Sperm features in turbot (Scophthalmus maximus): a comparison with other freshwater and marine fish species. Aquatic Living Resources 7:283-294.

Tabata, K. 1991. Application of the chromosomal manipulation in aquaculture of hirame Paralichthys olivaceus. Bulletin of the Hyogo Prefectural Fisheries Experimental Station 28:1-134.

Tabata, K. and A. Mizuta 1997. Cryopreservation of sex reversed gynogenetic female sperm in hirame. Fisheries Science 63:482-483.

Tabata, K., S. Gorie, and K. Nakamura 1986. Induction of gynogenetic diploid in hirame Paralichthys olivaceus. Bulletin of the Japanese Society of Scientific Fisheries 52:1901-1904.

Teletchea, F., J-N Gardeur, M. Psenicka, V. Kaspar, Y. Le Doŕe, O. Linhart, and P. Fontaine 2009. Effects of four factors on the quality of male reproductive cycle in pikeperch Sander lucioperca. Aquaculture 291:217-223.

Trippel, E.A. and J.D. Neilson 1992. Fertility and sperm quality of virgin and repeat-spawning Atlantic cod (Gadus morhua) and associated hatching success. Canadian Journal of Fisheries and Aquatic Sciences 49:2118-2127.

Veillette, P.A., M. Merino, N.D. Marcaccio, M.M. Garcia and J.L. Specker 2007. Cortisol is necessary for seawater tolerance in larvae of a marine teleost the summer flounder. General and Comparative Endocrinology 151:116-121.

Vermeirssen E.L.M, R.J. Sheilds, C. Mazzora de Quero, and A.P. Scott 2000. Gonadotropin releasing hormone agonist raises concentrations of progestins and enhances milt fluidity in Atlantic halibut (Hippoglossus hippoglossus). Fish Physiology and Biochemistry 22:77-87.

Viveiros, A.T.M., A. Jatzkowski, and J. Komen 2003. Effects of oxytocin on semen release Watanabe, W.O. and P.M. Carroll 2001. Progress in controlled breeding of summer flounder, Paralichthys dentatus, and southern flounder, P. Lethostigma. Journal of Applied Aquaculture 11:89-111.

Watanabe, W.O., E.P. Ellis, and S.C. Ellis 1998. Progress in controlled maturation and spawning of summer flounder Paralichthys dentatus broodstock. Journal of the World Aquaculture Society 29:393-404.

Yamamoto, E. 1999. Studies on sex-manipulation and production of cloned populations in hirame, Paralichthys olivaceus. Aquaculture 173:235-246.

Zohar, Y. 1996. New approaches for the manipulation of ovulation and spawning in farmed fish. Bulletin of the National Research Institute of Aquaculture Supplement 2:43-48.

Tables

Table 2.1. Parameter definitions for CASA measurements.

Parameter	Definition	Units
Duration (DUR)	Time from activation to zero percent motility of sample.	Sec
Path Velocity (VAP)	Average velocity of the smoothed cell path.	Mm/sec
Progressive Velocity (VSL)	Average velocity measured in a straight line from the beginning to the end of track.	Mm/sec
Track Speed (VCL)	Average velocity measured over the actual point-to-point track followed by the cell.	μm/sec
Linearity (LIN)	The departure of the cell track from a straight line. Average value of the ratio VSL/VCL.	
Motile percent (MOT)	Percent motile as defined by VSL and VAP cutoffs.	

Table 2.2 Volume and cell density of meiogyn and normal summer flounder sperm. Values that share a letter are not significantly different (t-test, $P < 0.05$). BW = fish body weight.

Treatment	Volume (ml)	Concentration (cells/ml x 10^9)	BW (kg)	Sperm (ml/kg BW)
Normal	2.0 ± 0.5^{a}	12.4 ± 1.6^{a}	1.1 ± 0.1^{a}	1.8 ± 0.4
Meiogynogen	0.3 ± 0.4^{b}	3.8 ± 1.2^{b}	0.4 ± 0.1^{b}	0.8 ± 0.3
	$p = 0.0068$	$p = 0.0002$	$p = 0.0001$	

Table 2.3. Spermatozoa characteristics of meiogyn and normal summer flounder measured by Computer-Assisted Sperm Analysis compared to those of other species. Spermatozoa characteristics indicated are duration (DUR), average path velocity (VAP), progressive velocity (VSL), track speed (VCL), linearity (LIN) and motile percent (MOT).

Parameter	Summer flounder meiogynogens	Summer flounder normal	Halibut[a]	Sea bream[b]	Hake[c]	Smelt[d]
DUR	328.5 ± 47.9	343.0 ± 43.7				
VAP	87.8 ± 4.1	104.3 ± 7.0		113.1	64.0	55.7
VSL	85.2 ± 3.9	100.8 ± 6.6	82.3		51.0	38.7
VCL	99.1 ± 4.0	115.1 ± 6.6	99.6		82.0	79.9
LIN	84.8 ± 1.1	86.5 ± 1.2	81.5			40.6
MOT	35.8 ± 5.8	49.5 ± 6.8	46.5	64.7		53.7

[a] Ottesen et al. 2009, [b] Liu et al. 2007, [c] Groison et al. 2010, [d] Krol et al. 2009

Table 2.4. Comparison of fertilization and hatching success using normal and meiogyn summer flounder sperm.

	Fertility Parameter (%)			
Treatment	Buoyancy	Fertilization	Pre-hatch	Larval viability
Normal	98 ± 0.0	47.0 ± 0.0	46.6 ± 0.0[a]	96.6 ± 3.3
Meiogynogenetic	97 ± 0.0	41.6 ± 0.0	36.3 ± 0.0[b]	98.3 ± 1.6
			P=0.0113	

Table 2.5 Fertility parameters in commercial production trials.

Female	Ovulated Eggs ($x10^3$)	Fertilization (%)	Incubator	Hatch (%)
1	275.9	90	1	45.2
2	77.9	70	1	
3	30	35	2	50.5
1	188.9	55	3	21.1
2	60	75	3	
3	40.7	NA	3	
4	67.2	NA	3	
3	95.9	40	4	
5	54	15	4	14
6	95.9	60	4	
7	95.9	55	4	

CHAPTER III

SPERMIATION INDUCTION IN SUMMER FLOUNDER (*PARALICTHYS DENTATUS*)

Introduction

In temperate fish species, reproductive development is usually stimulated by environmental cues, particularly and water temperature, that activate the hypothalamic-pituitary gonadal axis. Upon stimulation, gonadotropin releasing hormones (GnRHs), well conserved, 10 amino acid peptides are released from the hypothalamus and cause release of large glycoprotein gonadotropic hormones (follicle stimulating hormone and luteinizing hormone; FSH and LH) from the anterior pituitary (Zohar et al., 2010). These gonadotropins subsequently stimulate gonadal steroid production, necessary for normal gamete development, spawning behavior initiation, and fertilization. In captivity, however, many fish species fail to properly respond to simulated environmental conditions, and either undergo improper gamete development and/or do not initiate volitional spawning. The causes of reproductive failure in cultured fish are often unknown, but may be due to improper broodstock sex ratios, densities, tank size or spawning substrate.

In addition to manipulating environmental cues, researchers and culturists often use exogenous hormones to stimulate gamete development and/or initiate spawning behavior (Mylonas et al., 2010). Initially, preparations made from freeze-dried carp pituitaries (carp pituitary extract; CPE) were used for this purpose but were largely replaced with

the wide scale production of human chorionic gonadotropin (hCG). hCG is similar in structure to pituitary gonadotropins and has been used to stimulate ovulation in many fish species as well as domestic livestock. More recently, analogues of GnRHs have been used for fish broodstock, as their small size and structural similarity to native hormones were thought to be less likely to initiate immune responses than hCG or CPE. These analogues were found to be highly effective in inducing ovulation in many fish species. Although used less frequently than in females, exogenous hormones have also been used in male broodstock to stimulate spermatogenesis and spermiation. (Donaldson and Hunter, 1983, Lam, 1982, Zohar, 1989, Mylonas and Zohar 2001). As in females, GnRH analogues have been favored in recent years, and the use of slow release implants, eliminates the stress associated with frequent administration that are necessary with liquid injections. Male summer flounder often produce very small sperm volumes that can be limiting in commercial production (Cyr and Eales, 1996; Le Gac et al., 1993; Negatu et al., 1998; Weber et al., 1995).

The purpose of this study was to test the efficacy of several hormone preparations on spermiation in summer flounder to improve reproductive efficiency for commercial aquaculture.

Materials and Methods

Broodstock husbandry

Wild-caught and captive-bred (normal) summer flounder broodstock (> 7 years; females = 0.97-3.65 kg, males = 0.68-1.60 kg) were maintained at Great Bay Aquaculture LLC (GBA; Portsmouth, NH, USA) for at least four years prior to the start of the experiments.

The fish were implanted with a passive integrated transponder (PIT) tag (Biomark Inc., Boise, ID, USA) for individual identification and held in 6,000 - L fiberglass rectangular tanks incorporated in recirculating (28-30 ppt salinity) systems. The systems were equipped with biological and mechanical filtration, ultraviolet sterilization, foam fractionation, and photothermal control. Half-hour crepuscular periods were provided with 100 W incandescent bulbs to simulate dawn and dusk. Light intensity, measured with a light meter (Sper Scientific, Scottsdale, AZ, USA), ranged from 5 - lx (dawn/dusk) to 30 - lx (day) at the water surface during light period. Water temperature and dissolved oxygen were measured daily (Oxyguard, Birkerod, Denmark) and total ammonia nitrogen and nitrite were monitored weekly (HACH®; Loveland, CO USA). Water quality within the culture tanks remained within ranges suitable for rearing this species (Watanabe et al. 1998). The fish were fed a commercial ration (9 - mm pellet, 54% protein, 18% fat; Vitalis Cal, Skretting, Trouw, Spain) to apparent satiation 2-3 times per week. As summer flounder naturally spawn in the fall, and temperature were maintained at 12L: 12D and 19 ± 1 C until two months prior to desired spawning and then gradually adjusted to to 8L: 16D and 14 C, respectively (Watanabe et al., 1998; Bengtson ,1999).

Meiogyn production

Meiogynogenetic fish (0.24-0.77 kg) were produced as reported previously (Colburn et al., 2009). Briefly, summer flounder eggs were activated with ultraviolet-irradiated black sea bass, *Centropristis striata* sperm, and a 6 - min pressure shock (8,500 psi) was applied 2 min post-fertilization. The eggs and larvae were incubated (16-17° C and 35 ppt) and reared (16-18 ° C and 27-31 ppt) under standard hatchery conditions and

fed enriched rotifers followed by *Artemia*. Upon metamorphosis (41 days-post hatch; DPH), the juveniles (n = 900) were transferred to 235 - L cylindrical tanks that were incorporated into recirculating systems and subjected to male-determining temperatures ($\geq$ 21 C). At 376 DPH, 153 of the remaining fish were transferred to the broodstock systems described above, and held for approximately 3 years prior to the start of the experiments.

Induction

Six groups of six male, meiogynogen summer flounder between 0.2 and 0.5 kg were injected with six different hormonal treatments to induce spermiation. Before application of the treatments, milt was extracted by gentle abdominal massage. Individual fish were selected for this experiment by their milt and spermatozoa characteristics. To qualify for this experiment all of the males must have produced between 0.05ml and 0.3ml of milt during initial extraction. Additionally, spermatozoa motility was graded by a qualitative scoring system. Before use, sperm (diluted 1:50; 1 µl milt : 49 µl seawater) were checked for motility with a compound microscope immediately after activation with ultraviolet-sterilized seawater (30 g L^{-1}). Motility was estimated with an arbitrary scale, ranging from 0-4, where 0 represents no motility; 1, 1- 25%; 2, 26-50%; 3, 51-75%; and 4, 76-100% motile sperm (Viveiros et al., 2003).

Of the treatments used, four were active hormones and two were controls. The treatments included carp pituitary extract (CPE, 2 mg kg^{-1}; Stoller Fisheries, Spirit Lake, IA, USA; Smigielski, 1975; Berlinsky et al., 1997), liquid gonadotropin releasing hormone (GnRH) at 25 µg/kg bodyweight, human chorionic gonadotropin (HCG) at 600 IU/kg bodyweight,

and a cholesterol/cellulose implant containing 25 μg GnRH. The control treatments included a 32 mg cholesterol/cellulose sham implant and liquid physiological saline (0.9%) injected at 550 μL/kg BW. All of the liquid treatments were injected daily for two weeks. The implanted treatments were administered at days one, seven and fourteen of the experiment. At day eight and fifteen all of the fish were checked for milt production by gentle abdominal massage. Milt was collected in 2 mL polypropylene microcentrifuge tubes and held on ice until motility could be determined.

Results

Milt volume

No significant differences in volume were seen in any induction treatment for initial or day 8 milt collections. Fish treated with CPE produced significantly greater volumes than fish treated with a sham implant on day 15 (Fig. 3.1).

Spermatozoa counts

No significant differences in total spermatozoa produced by induction treatment were seen between fish at day 8. A significant increase in spermatozoa produced by GnRH liquid injection was seen at day 15 compared to sham implant (Fig 3.2).

Assessing sperm Motility

Motility of meiogynogenetic sperm was compared for the duration of the study. Motility was assessed at the start of the study and at days 8 and 15. No significant differences were found in any of the treatments between days of motility assessment (Fig. 3.3).

Discussion

Sperm density and volume vary considerably among teleost species and are further influenced by season, age and frequency of stripping. Summer flounder are among several Pleuronectiform species that produce relatively low volumes of concentrated, high-density sperm (Lanes et al., 2010). Expressible volumes less than 1 mL were also reported for turbot (*Psetta maxima*; Suquet et al., 1994), yellowtail flounder (*Limanda ferruginea*) (Clearwater and Crim, 1998), and Brazilian flounder (*Paralichthys orbignyanus*) (Lanes et al., 2010) that produce relatively low volumes of (concentrated, high density sperm). Although direct comparisons with normal males of the same age were not available, production from the meiogynogenetic summer flounder used in the present study may have been further reduced because of reduced body mass, likely associated with increased homozygosity.

hCG and analogues of GnRH have been used to accelerate the onset of spermiation as well as increase milt production in a number of teleost species but considerable species-specific differences in responsiveness have been reported (Mylonis and Zohar, 2001). For instance, analogues of GnRH have been shown to accelerate spermiation in Atlantic halibut (*Hippoglossus hippoglossus*; Vermeirssen et al. 2004) and golden rabbitfish (*Siganus guttatus*; Komatsu et al. 2006), but were ineffective in Japanese eels (*Anguilla japonica*; Kagawa et al., 2009) that responded to hCG and salmon pituitary extract. Similar results were found with hCG, which despite wide-spread effectiveness (e.g. European sea bass (*Dicentrachus labrax*; Schiavone et al. 2006), pejerrey (*Odontesthes bonariensis*; Miranda et al., 2005) and New Zealand

snapper (*Pagrus auratus*; Pankhurst, 1994) was ineffective on European smelt (*Osmerus eperlanus*; Król et al., 2009) which responded to GnRH analogues. Some of the factors that have been shown to influence species-specific responsiveness include administration method and dose, fish age, spawning season stage, frequency of stripping and degree of dopaminergic inhibition (Büyükhatipoglu and Holtz, 1984; Vermeirssen et al., 2004; Kagawa et al., 2009). Although currently used less frequently, pituitary extracts (carp and salmon) are also used as spermiation-induction agents (Miranda et al., 2005; Kagawa et al., 2009). It was previously shown that GnRH, hCG and CPE were all ineffective in inducing spermiation in non-spermiating summer flounder (Berlinsky et al., 1997). Although GnRH administered as an injectable liquid increased spermiation in the present study, the extent to which it did so wasn't as pronounced as that reported in some other studies (Miranda et al., 2005; Rzemieniecki et al., 2004) and milt volumes remained very small. Additional studies are necessary to determine if low milt production is a function of genotype (gynogenetic) or sub-optimal induction regimes for this species.

Exogenously administered hormones were found to stimulate spermiation in as little as 24 hrs in some species (Miranda et al., 2005; Król et al., 2009; Rzemieniecki et al., 2004), but longer administration periods (21-42 days) were examined in other species (Lim et al., 2004; Schiavone et al., 2006). In European sea bass (*Dicentrarchus labrax*) and greenback flounder (*Rhombosolea tapirina*) administered hCG and GnRHa implants, respectively, milt volume increased by one week post-treatment and typically remained elevated for a week thereafter (Lim et al., 2004; Schiavone et al., 2006). The timeframe for spermiation induction in the present study was based on industry practices

where summer flounder broodstock are typically administered CPE daily for up to 14 days to induce ovulation and spermiation (G. Nardi personal Communication; Bengton, 1999; King et al., 2001).

In the present study milt volume increased from days 8-14 with repeated CPE injections. These results are consistent with those of Viveros (2002), who reported additive effects of CPE administration on milt hydration in African catfish, *Clarias gariepinus*. As increases in milt volume were observed between the days 7 and 14 sampling, it is possible that further increases could have occurred if the study were conducted for a longer duration. Considering the labor involved and stress associated with daily injections, further experiments with long acting hormone implants may be warranted.

References

Bengtson, D.A., 1999. Aquaculture of summer flounder (*Paralichthys dentatus*): Status of knowledge, current research and future research priorities. Aquaculture 176, 39-49.

Berlinsky, D.L., King, W.V., Hodson, R.G., Sullivan, C.V., 1997. Hormone induced spawning of summer flounder, *Paralichthys dentatus*. Journal of the World Aquaculture Society 28, 79-86.

Büyükhatipoglu, S., Holtz, W., 1984. Sperm output in rainbow trout (*Salmo gairdneri*)—effect of age, timing and frequency of stripping and presence of females. Aquaculture 37, 63-71.

Clearwater, S.J., Crim, L.W., 1998. Gonadotropin releasing hormone-analogue treatment increases sperm motility, seminal plasma pH and sperm production in yellowtail flounder, *Pleuronectes ferrugineus*. Fish Physiology and Biochemistry 19, 349-357.

Cyr D.G., Eales J.G., 1996. Interrelationships between thyroidal and reproductive endocrine systems in fish Reviews in Fish Biology and Fisheries. 6, 165-200.

Donaldson E.M., Hunter GA., 1983. Induced final maturation, ovulation, and spermiation in cultured fish. Fish Physiology: Vol. 9: Reproduction, Pt. B: Behavior and Fertility Control. Academic press. pp.351

Kagawa H., Kasuga Y., Adachi J., Nishi A., Hashimoto H., Imaizumi H., Kaji S., 2009. Effects of continuous administration of human chorionic gonadotropin, salmon pituitary extract, and gonadotropin-releasing hormone using osmotic pumps on induction of sexual maturation in male Japanese eel, *Anguilla japonica*. Aquaculture 296, 117-122.

King N.J., Nardi G.C., Jones C.J. 2001. Sex linked growth divergence of summerflounder from a commercial farm: are males worth the effort? J. Appl. Aquac., 11, 77–88

Krol, J., Kowalski, R.K., Hliwa, P., Dietrich, G.J., Stabinski, R., Ciereszko, A., 2009. The effects of commercial preparations containing two different GnRH analogues and dopamine antagonists on spermiation and sperm characterization in the European smelt, *Osmerus eperlanus* (L). Aquaculture 286, 328-331

Lam T.J., 1982. Applications of endocrinology to fish culture. Canadian Journal of Fisheries and Aquatic Sciences 39. 111-137.

Lanes, C.F.C., Okamoto, M.H., Bianchini, A., Marins, L.F., Sampaio, L.A., 2010. Sperm quality of Brazilian flounder, *Paralichthys orbignyanus,* throughout the reproductive season. Aquaculture Research 41, 199-207.

Le Gac F., Blaise O., Fostier A., Le Bail P-Y., et al., 1993. Growth hormone (GH) and reproduction: A review. Fish Physiology and Biochemistry 11, 219-232.

Lethimonier C., Madigou T., Munoz-Cueto J., Lareyre J., 2004. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. General and Comparative Endocrinology 135,1-16.

Lim H.K., Pankhurst N.W., Fitzgibbon Q.P., 2004. Effects of slow release gonadotropin releasing hormone analog on milt characteristics and plasma levels of gonadal steroids in greenback flounder, *Rhombosolea tapirina*. Aquaculture 240, 505-516.

Maria A.N., Azevedo H.C., Santos J.P., Silva CA., Carneiro P.C.F., 2010. Semen characterization and sperm structure of the Amazon tambaqui *Colossoma macropomum*. Journal of Applied Ichthyology 26, 779-783.

Miranda L.A., Cassara M.C., Somoza G. M., 2005 Increase in milt production by hormonal treatment in the pejerrey fish, *Odontesthes bonariensis* (Valenciennes 1835). Aquaculture Research 36, 1473-1479.

Mylonas C.C., Fostier A., Zanuy S., 2010. Broodstock management and hormonal manipulations of fish reproduction. General and Comparative Endocrinology 165, 516-534.

Mylonas, C.C., Zohar, Y., 2001. Endocrine regulation and artificial induction of oocyte maturation and spermiation in basses of the genus *Morone.* Aquaculture 202,. 205-220.

Negatu Z., Hsiao S.M., Wallace R.A ., 1998 Effects of insulin-like growth factor-I on final oocyte maturation and steroid production in *Fundulus heteroclitus.* Fish Physiology and Biochemistry 19, 13-21

Pankhur N.W., 1994. Effects of gonadotropin releasing hormone analogue, human chorionic gonadotropin and gonadal steroids on milt volume in the New Zealand snapper, *Pagrus auratus* (Sparidae). Aquaculture 125, 185-197.

Rzemieniecki A., Domagalla J., Glogowski J., Ciereszko A., Trzebiatowski R., Kouil J., Hamackova J., Babiak I., 2004. Induced spermiation in 3-year-old sterlet, *Acipenser ruthenus* L. Aquaculture Research 35, 144-151.

Smigielski, A.S., 1975. Hormone-induced spawnings of the summer flounder and rearing of the larvae in the laboratory. The Progressive Fish-Culturist 37, 3-8.

Suquet, M., Billard, R., Cosson, J., Dorange, G., Chauvaud, L., Mugnier, C., Fauvel, C., 1994. Sperm features in turbot (*Scophthalmus maximus*): a comparison with other freshwater and marine fish species. Aquatic Living Resources 7, 283-294.

Vermeirssen E.L., Mazorra de Quero C., Shields R.J., Norberg B., Kime D.E., Scott A.P., 2004. Fertility and motility of sperm from Atlantic halibut (*Hippoglossus hippoglossus*) in relation to dose and timing of gonadotrophin-releasing hormone agonist implant Aquaculture 230, 547-567.

Viveiros A. T. M., Fessehaye Y., Ter Veld M., Schulz R.W., Komen J., 2002. Hand stripping of semen and quality after maturational hormone treatments, in African catfish *Clarias gariepinus.* Aquaculture 213, 373–386.

Viveiros, A.T.M., Jatzkowski, A., Komen, J., 2003. Effects of oxytocin on semen release response in African catfish (*Clarias gariepinus*). Theriogenology 59, 1905-1917.

Zohar, Y., Munoz-Cueto, J, A., Elizur, A., Kath, O., 2010. Neuroendocrinology of reproduction in teleost fish. Gen.Comp. Endocrinology 165, 438-455

Zohar, Y., 1989. Fish reproduction: Its physiology and artificial manipulation. CRC Press, Boca Raton, FL.

Tables and Figures

Table 3.1 Concentrations and administration technique of hormones applied during the experiment.

Treatments	Concentration	Administration
$GnRH_a$ implant	25 μg/implant	Implanted day 1 and day 8
$GnRH_a$ injection	25 μg/ml injected 0.5ml/kg body weight	Intramusculature injection daily for 15 days
Carp pituitary extract (CPE)	2 mg/ml injected 0.5ml/kg body weight	Intramusculature injection daily for 15 days
Saline injection	0.9% saline injected 0.5ml/kg bodyweight	Intramusculature injection daily for 15 days
Human chorionic gonadotropin (hCG)	600 IU/kg body weight	Intramusculature injection daily for 15 days
Sham Implant	Cholesterol/cellulose implant	Implanted day 1 and day 8

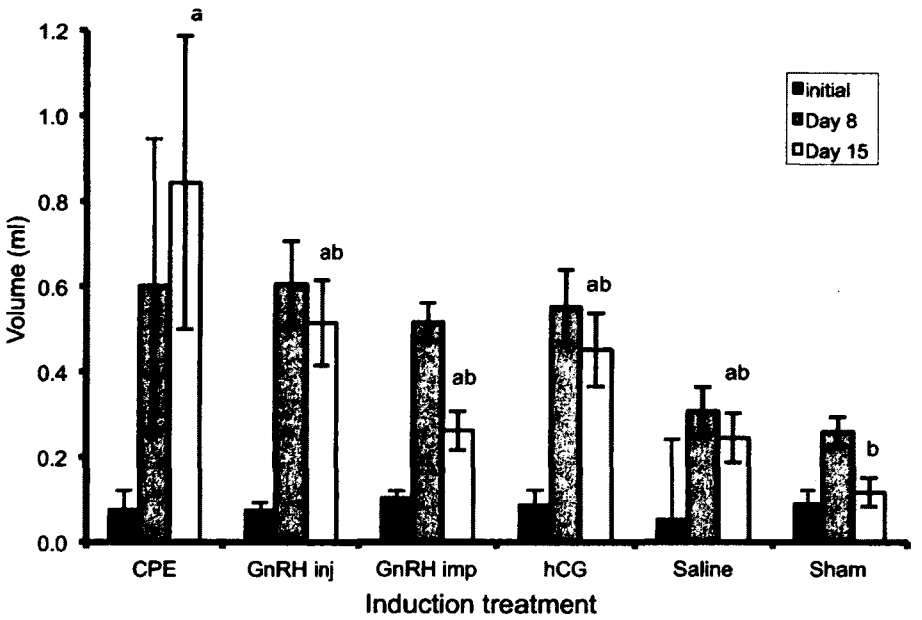

Figure 3.1.

Mean values (± SEM) for milt volume calculated for induction treatments. Initial, Day 8 and Day 15 volumes were obtained from milt collected initially before treatment and on the eighth and fifteenth day of the experiment. Values labeled by the same letter are not significantly different (P>0.05).

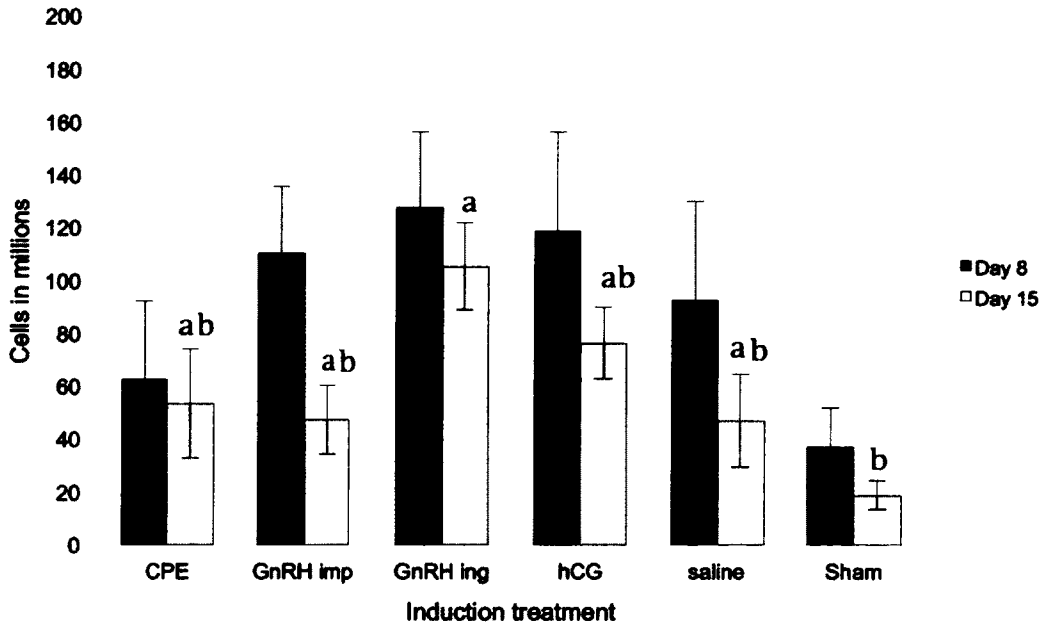

Figure 3.2.

Mean values (± SEM) for total cell counts calculated for induction treatments. Day 8 and Day 15 cell totals were obtained from milt collected on the seventh and fourteenth day of the experiment. Columns labeled by the same letter are not significantly different ($P>0.05$).

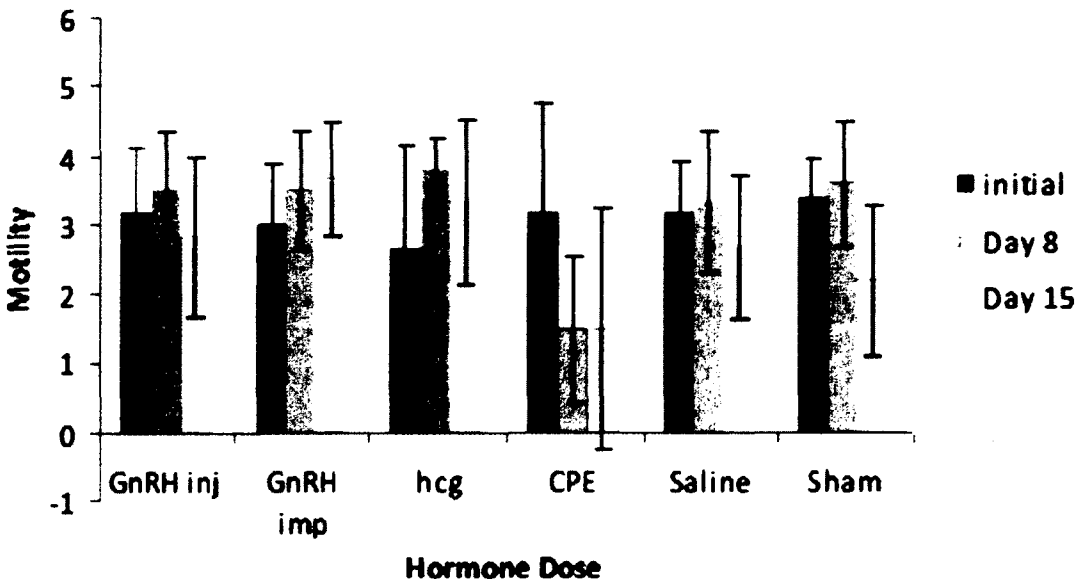

Fig. 3.3

Mean motility values for each hormone treatment. Motility was measured initially before treatment application and at day 8 and day 15 of the experiment. Motility was assessed using an ordinal scoring system based on (Viveiros et al., 2003)

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

The experiments that I conducted for my thesis research represent important steps toward controlling reproduction in summer flounder and creating monosex (all female) populations.

The cryopreservation studies revealed that fertilization and hatching were not different in trials using fresh or cryopreserved sperm at a slow freezing rate -5 deg C/min^{-1}, DMSO cryoprotectant and a sucrose based extender. These experiments determined that the parameters suitable for freezing summer flounder sperm (*Paralichtys olivaceus)* were similar to related species, Japanese flounder (*Paralichthys olivaceus)* and Brazillian flounder (*Paralichthys orbignyanus*). It may be possible to achieve greater fertilization and hatching if the cryopreservation protocol were further optimized, this would require varying the parameters used in the cryopreservation protocol such as, freezing rate, cryoprotectant type and concentration, dilution ratio, and extender composition. Finally, these experiments observed the post thaw viability of sperm after a short duration of storage (approximately 24 hours). Further experiments could evaluate the viability of sperm stored over a greater period of time (weeks or months).

While comparing sperm from normal and meiogynogenetic summer flounder, I determined that the two were similar when analyzed by CASA but lower embryonic

viability was observed with the use of sperm from meiogynogenetic males. Examination of the genetic composition of meiogyn sperm may reveal what specific abnormalities may be causing the decreased fertility and developmental dysfunction. Possible causes of dysfunction may be that meiogyn males have a greater number of deleterious alleles associated with an increased homozygosity compared to normal male flounder.

Further experimentation will be necessary to determine the effects of temperature and other environmental factors (e.g. density, substrate, lighting, nutrition) on sex determination and differentiation in this species, as it has been shown in congeneric species that female genotypes (e.g. XX) will only result in female phenotypes if correct environmental conditions are applied.

APPENDICES

University of New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
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22-Sep-2011

Berlinsky, David L
Biological Sciences, Rudman Hall
Durham, NH 03824

IACUC #: 091006
Project: Increasing Production and Cytopreservation of Summer Flounder Sperm
Category: C
Next Review Date: 16-Oct-2012

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your request for a time extension for this protocol. Approval is granted until the "Next Review Date" indicated above. You will be asked to submit a report with regard to the involvement of animals in this study before that date. If your study is still active, you may apply for extension of IACUC approval through this office.

The appropriate use and care of animals in your study is an ongoing process for which you hold primary responsibility. Changes in your protocol must be submitted to the IACUC for review and approval prior to their implementation.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact me at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,