TERMINATION REPORT

SIGNATURE PAGE

PROJECT CODE: 03-1 **SUBCONTRACT NO: 557005**

PROJECT TITLE: Development of culture methods for commercial production of rainbow smelt (Osmerus mordax)

PREPARED BY: Dr. David Berlinsky

2/28/06

Project Coordinator or PI Date

TERMINATION REPORT

PROJECT CODE: 03-1 **SUBCONTRACT/ACCOUNT NO:557005**

PROJECT TITLE: "Development of Culture Methods for Commercial Production of Rainbow Smelt (*Osmerus mordax*)"

DATES OF WORK: 1/1/03-12/30/05

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REASON FOR TERMINATION: Objectives completed

PROJECT OBJECTIVES:

1) Optimize environmental conditions for intensive production of rainbow smelt larvae.
2) Investigate pond culture methodologies that promote high survival and growth of rainbow smelt larvae
3) Determine the optimal feeding progression of prey species conducive for larval smelt survival
4) Develop a practical protocol for rearing rainbow smelt larvae for commercial production
5) Teach established and prospective aquaculturists techniques for profitable smelt culture for development of a new industry

ANTICIPATED BENEFITS:

The goal of this project is to develop specific culture methods for rainbow smelt in order to help establish an industry centered in the Northeast. Commercial culture of rainbow smelt would: 1) reduce fishing pressures on wild stocks, 2) provide economic opportunities for established and novice culturists and 3) increase the available supply of rainbow smelt to bait dealers to meet existing demand. In addition, techniques developed for culture of rainbow smelt will likely be transferable to other species, including the threatened delta smelt (*Hypomesus transpacificus*).

PRINCIPLE ACCOMPLISHMENTS

Objective 1- Optimize environmental conditions for intensive production of rainbow smelt larvae. Complete details of the findings for this objective are presented in Part II, Technical Analysis and Summary.

Broodstock were collected by fyke net from the Exeter River during their annual spawning migrations in March 2003-05. The fish were transported to the University of New Hampshire and strip-spawned, without hormone induction, within 5 d of capture according to our previously published methods (Ayer et al. 2005). To determine the effects of salinity and temperature on hatching success, fertilized eggs were cultured in sterile Petrie dishes under controlled conditions. Eggs were incubated at 5, 10, 15 and 20 C at salinity levels of 0, 10, 20 and 30 ppt. For larval experiments, the embryos were cultured in 2 l MacDonalds's jars until hatching occurred. Upon hatching, larvae were assigned to experiments to assess the effects of salinity, temperature, addition of microalgae and light intensity (100 vs. 500 lx) on growth and survival.

Our data indicated that hatching occurs over a range of temperatures from 5-20° C but was greatest at 10 and 15 °C. No hatching occurred at 20 or 30 ppt but was not different at 0 or 10 ppt. Larval survival was significantly impacted without the addition of microalgae to the culture water (87% survival vs. 17%) but not by light intensity. Growth was greater at 20 vs. 15 °C and declined with higher salinity concentrations (20 < 10 < 2 ppt and 8 < 5 < 2 ppt).

Objective 2. Investigate pond culture methodologies that promote high survival and growth of rainbow smelt larvae.

In April-June, 2003-05 broodstock were harvested from the Kennebec River, transported to Harmon Brook Farm (Canaan Maine) and spawned as above. On average, twenty thousand larvae (5 DPH) were stocked in 6-0.2 hectare ponds that had been enriched for zooplankton growth and rid of predatory insects. Survival of smelt was extremely variable among ponds ranging from 0 to approximately 2000. The reason for the wide variability was likely due to prey availability. Upon harvest in the fall (Oct-November) smelt were 60-110 mm in length.

Objective 3. Determine the optimal feeding progression of prey species conducive for larval smelt survival.

In the laboratory, we determined that at 10-15°C larval smelt begin feeding on marine rotifers (*Brachionus plicatilis*) at ~3 days post-hatch (DPH) and will accept *Artemia* nauplii beginning approximately 28 DPH. At 60 DPH larvae began accepting commercially prepared diets (400 μm). In pond culture, the smelt larvae primary preyed upon freshwater rotifers at the start followed by daphnia. Consumption of commercial prepared diet variable.

Objective 4. Develop a practical protocol for rearing rainbow smelt larvae for commercial production.

We developed methodologies for spawning wild-caught smelt with extremely high levels of fertilization (>95%) and hatch (>80%). This accomplishment was made possible primarily by eliminating bacterial and fungal infections that plagued earlier attempts by removing the adhesiveness of the eggs prior to incubation. This step permitted frequent water changes and sufficient aeration necessary high embryonic survival. Additionally we developed culture conditions (temperature, salinity, greenwater) and feeding protocols for commercial scale production larval production.

Objective 5. Teach established and prospective aquaculturists techniques for profitable smelt culture for development of a new industry

The methodologies developed during these studies were used by our industry partner (John Whalen) for the first successful sales of farm reared smelt as bait for recreational fishing. Interest in raising smelt has been expressed, by a number of novice culturists (including our non-funded participants, Mr. Dick Wollmar and Joseph Vaillancourt, and others (e.g. Mr. Lou Newski, Berwick, ME)) through our

outreach efforts. The results were presented at aquaculture meetings ("Development of practical culture methods for rainbow smelt *Osmerus mordax*"- M. Ayer and D.L. Berlinsky Northeast Aquaculture Conference and Exposition. December 4, 2004, Manchester NH) as well as in scientific journals. Ms. Julie Newman coordinated outreach activities with all interested baitfish farmers.

IMPACTS:

1) Working protocols for commercial-scale hatchery production of rainbow smelt were developed.
2) Results were presented in peer-refereed scientific publications and aquaculture meetings
3) Sales of farm-raised smelt were initiated in 2005.
4) Mr. John Whalen, our industry collaborator submitted a Small Business Innovative Research proposal to the USDA, to further commercialization of smelt culture
5) Additional growers are initiating smelt culture

RECOMMENDED FOLLOW-UP ACTIVITIES

Future studies should explore nutritional needs of juvenile smelt through the growout phase as well as harvesting and transport methodologies.

SUPPORT:

YEAR	NRAC-USDA FUNDING	OTHER SUPPORT					TOTAL SUPPORT
		UNIVER-SITY	INDUSTRY	OTHER FEDERAL	OTHER	TOTAL	
1	$126,208						$126,208
TOTAL	$126,208						$126,208

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:

M.H. Ayer, C. Benton, W. King V, J..Kneebone, S. Elzey, M. Toran, K. Grange and D.L. Berlinsky. Development of Practical Culture Methods for Rainbow Smelt Osmerus mordax Larvae. North American Journal of Aquaculture. 2005. 67:202-209.

J.D. DeGraaf and D.L. Berlinsky. Cryogenic and refrigerated storage of Rainbow smelt (Osmerus mordax) spermatozoa 2004. Journal of the World Aquaculture Society. 35:(2) 244-231.

PART II

The following manuscripts were published as reported under "PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED" above.

Development of practical culture methods for rainbow smelt larvae

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Running title: culture of smelt larvae

Abstract

Wild caught rainbow smelt *Osmerus mordax*, collected and strip spawned during the 2002 and 2003 spawning seasons, produced fertilization and hatching rates ranging from 60-99%. During the 2002 spawning season, embryos were stocked into petri dishes (n = 96) and the effects of temperature (5, 10, 15, and 20°C) and salinity (0, 10, 20, and 30 ppt) on hatching success were determined. Hatching was significantly greater ($P < 0.05$) at 10 and 15°C when compared to fish at 5 and 20°C

regardless of salinity. No hatching occurred at 20 or 30 ppt salinity at any temperature. In separate experiments, the effects of light intensity, green water (200,000 microalgal cells/mL) temperature and salinity on larval growth and survival were tested. Growth and survival were significantly improved by the addition of green water, but no significant differences were found between light intensity treatments. In studies conducted during the 2003 spawning season, there was no difference in survival between smelt larvae reared at 15 or 20°C or among salinity levels of 2, 10, or 20 ppt. Larval growth was inversely proportional to salinity level. As salinity levels decreased, larval growth rates increased significantly (2 > 10 > 20 ppt). Larvae reared for 77 d at 20°C fed sequentially on enriched rotifers, *Artemia* nauplii and a commercial marine finfish diet. The results from this study suggest that rainbow smelt are tolerant of a wide range of environmental conditions and are a good baitfish candidate for culture in the northeast USA.

Introduction

The rainbow smelt *Osmerus mordax* is enjoyed as a food fish, particularly in the northeastern U.S. and appears in fish markets, grocery stores, and restaurants (Klein-MacPhee 2002). Smelt are the preferred prey of landlocked Atlantic salmon *Salmo salar* during the winter months and are also targeted by other game species (Sayers et al. 1989). For this reason rainbow smelt, when available, are often the preferred bait of anglers.

During ice breakup in the spring, rainbow smelt migrate upstream from estuaries and coastal areas into freshwater streams and spawn at night when water temperatures reach 4 - 9°C. Spawning occurs in late February - March in Massachusetts and from late May - June in Maine and Canada (Bigelow

and Schroeder 1953; Crestin 1973; Lawton et al. 1990). The demersal eggs (~1.0 mm in diameter) adhere to rocks, sticks or grass and hatch in 1 - 4 weeks depending on water temperature (McKenzie 1964). Smelt larvae are about 5 mm at hatching and are carried downstream to brackish water.

Populations of anadromous smelt have been in decline in the northeastern United States for decades and availability of live, wild-caught fish is often limited. Human activities in the coastal zone have been implicated in the decline of many anadromous species, including smelt (Murawski and Cole 1978). Declines in smelt abundance in Massachusetts have been linked to industrial pollution, blockage of spawning migrations by dams, and the loss of eelgrass beds (Crestin 1973). It has been reported that obstructions in rivers, such as natural log dams, have prevented smelt from reaching their preferred spawning locations (Klein-MacPhee 2002).

Due to declines in wild rainbow smelt populations and their high value as a baitfish ($73-100/kg; Kircheis and Elliot 1989), there is enormous potential for culture to supply the live bait market. Available information on the spawning and larviculture of this species is extremely limited, however, and is derived primarily from a single published report (Akielaszek et al. 1985). Results from that study demonstrated that smelt larvae would consume salt-water rotifers *Brachionus plicatilis*, and when grown in ponds, attained marketable size in a single growing season. The purpose of the present study was to establish practical methods for smelt larviculture for commercial production.

Materials and Methods

Broodstock collection and spawning

In March 2002, adult smelt (n = 43; 34.4 ± 1.8 g) were collected through the ice by hook and line from the Exeter River in Exeter, New Hampshire. The fish were transported to the University of New Hampshire's Aquaculture Research Center (ARC) and placed into two 151-L glass aquaria containing non-chlorinated fresh water (NCFW; 10°C) and supplemental aeration. The fish were strip spawned

within 5 d of capture. Gravid smelt (n = 30) were also collected in a fyke net from the Exeter River during their annual spawning migration in March 2002. The fish were transported in aerated, fresh, river water to the ARC and spawned immediately, as described below. Females weighed 37 ± 2.6 g (mean ± SE) while males weighed 32 ± 4.2 g.

In April 2003, adult smelt were collected by dip net during their annual spawning migration in a tributary of the Damariscotta River in Damariscotta Maine (n = 25; 38.1 ± 3.2 g). The fish were transported to the ARC and placed into two 151-L glass aquaria, containing non-chlorinated fresh water (NCFW; 10°C) and supplemental aeration, and strip spawned within 5 d of capture.

Milt was collected by abdominal massage into plastic transfer pipettes and held on ice in 100-mL glass beakers for less than 30 min. Sperm motility was confirmed by microscopic examination (Olympus CH-2 Melville, New York, 400X) following activation with NCFW. Eggs were stripped into a polystyrene plastic dish using slight abdominal pressure. Fecundity (number of eggs/g body weight) was determined by weighing the fish, and then the expressed eggs, to the nearest 0.01 g. The mean number of eggs in three-1 g aliquots was then counted using a dissecting microscope. The eggs from each spawn were fertilized with 200-µL of pooled milt obtained from 2-3 males and then activated with 25-mL of NCFW. The gametes were gently swirled for 3 min, poured slowly into a tannic acid solution (150 ppm) and then swirled for an additional 10 min to remove adhesiveness (Rottman et al. 1988). Eggs from each spawn (2-6 females) were pooled, sieved (300 µm), rinsed with NCFW and incubated in a hatching jar with NCFW and supplemental aeration at 10°C. One batch of fertilized eggs was treated with 50 ppm P.V.P. iodine (Providone Iodine, 10%; Western Chemical, Ferndale, Washington) as a prophylactic for fungal infection. Fertilization success from all spawns was verified with a dissecting microscope by examining 100 embryos for the presence or absence of cleavage 24-h post-fertilization. Embryo development was monitored daily and pre-hatch viability was calculated by examining 200 embryos microscopically < 24 h prior to hatching. Hatched larvae were used for larval growth and survival and growth studies as described below.

Hatch experiment

Ten embryos from Exeter River broodstock (5-h post-spawn) were transferred by pipette to each of 96 sterile petri dishes (100 x 15 mm) containing 100-mL NCFW mixed with autoclaved seawater to achieve salinity levels of 0, 10, 20, and 30 ppt. Salinity was confirmed with a refractometer (Spartan Refractometers, Tokyo, Japan). Using a full 2 factor experimental design, embryos at each salinity level were tested at each of four temperatures (5, 10, 15, and 20 ± 1°C) and each salinity/temperature treatment was replicated six times. Embryos were incubated in controlled-temperature rooms without lights and monitored daily for evidence of hatching. The experiment was terminated when unhatched embryos appeared necrotic.

Feeding regimen

To establish a standardized feeding regimen for the experiments, approximately 1000 1-d post-hatch (DPH) larvae, from Exeter River broodstock , were transferred to each of two 151-L aquaria containing brackish water (10 ppt) and reared in a controlled temperature room at 20 ± 1°C**.** Overhead fluorescent lights provided continuous light (~500 lx at water surface) and light intensity was measured with an Extech™ light meter (Extech Instrument Co., Waltham, Massachusetts). The micro algae, *Nannochloropsis occulata,* (green water; 200,000 microalgal cells/mL; GW) were added to the tanks and their density was determined spectrophotometrically at 630 nm (King 1999). To reduce waste buildup, 20% of the water was replaced at 2-d intervals by siphoning. After the water exchange, algae were added to maintain the target density. Beginning 2 DPH, marine rotifers *Brachionus plicatilis* enriched in concentrated *N*. *occulata* for 8 h were added to the culture system. The rotifers were maintained at 10/mL in culture tanks by daily enumeration and supplementation.

At 28 DPH, the larvae were offered *Artemia* nauplii (Salt Creek™ Salt Lake City, Utah) in addition to enriched rotifers. At 32 DPH, rotifer and green water supplementation were discontinued. At 60 DPH, the larvae were offered a commercially-prepared diet (Biokyowa 400, Tokyo, Japan) in addition to *Artemia*. At 63 DPH, *Artemia* supplementation was discontinued and the larvae received only the Biokyowa diet for the remainder of the experiment (77 DPH). At 3-d intervals, water quality parameters (dissolved oxygen, nitrite, TAN, and pH) were tested using a commercial kit (Hach™ Model #FF-1A, Loveland, Colorado). Exceptions to these conditions are indicated in the descriptions for each experiment.

Effect of green water on growth

One-DPH larvae, from Exeter River broodstock , were maintained under standard conditions (see Feeding Regimen) prior to the start of this experiment. At 8 DPH, 100 larvae were transferred to each of six 38-L aquaria containing NCFW mixed with autoclaved seawater (15 ± 1°C, 10 ppt salinity). Three tanks contained GW and enriched rotifers at standard densities (10 rotifers/mL) while the remaining three tanks received enriched rotifers alone. After 14 d (22 DPH), surviving fish from each tank were enumerated and six larvae from each tank were measured to the nearest 0.1 mm using a dissecting microscope and an occular micrometer.

Effect of light intensity on growth

One-DPH larvae, from the Exeter River broodstock , were raised under standard conditions through 28 DPH. At 29 DPH, 200 larvae were transferred to each of six 38-L tanks containing NCFW mixed with autoclaved seawater (15 ± 1°C, 10 ppt salinity). The sides of each aquarium were wrapped in black polyethylene sheeting. Three of the tanks were covered with clear plexi-glass so they received continuous overhead fluorescent light (~500 lx at water surface) while the remaining three tanks were covered with plexi-glass wrapped in white bench paper which provided ~100 lx at water surface. Algal-enriched rotifers (10/mL) and newly hatched *Artemia* nauplii (5/mL) were provided during the first 7 d

of the experiment. From day eight (37 DPH) until the conclusion of the experiment, larvae were fed only *Artemia* nauplii (10/mL). The experiment was terminated after 14 d, the surviving larvae from each tank were enumerated and the lengths of 12 larvae from each tank were measured as described above.

Effects of temperature and salinity on growth

Two experiments were conducted on two different occasions using the same temperatures but different salinities. In experiment 1, 2000 1-DPH larvae, from the Damariscotta River broodstock 2003, were maintained in a 151-L aquarium under standard conditions. At 5 DPH 300 larvae were transferred to 18 38-L aquaria containing 10 ppt salinity water at 15 ± 1°C water. The salinity was adjusted 5 ppt/day to 2, 10, and 20 ppt. Once the larvae had acclimated to the increased salinity, nine aquaria were transferred to a 20°C controlled temperature room (full 2 factor experimental design). Larvae were fed enriched rotifers (10 rotifers/mL) and water quality was monitored as described above. After 10 d, the surviving larvae from each tank were enumerated and six larvae from each aquarium were measured to the nearest 0.01 mm using a Macintosh computer, the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) and a dissecting microscope. Experiment 2 was conducted with fish of the same age from a subsequent spawn, from the Damariscotta River broodstock 2003, using the same temperatures (15 and 20°C) but salinity levels of 2, 5, and 8 ppt.

Data analysis

Data are expressed as the mean ± SE and were analyzed by ANOVA using Systat 10 software (Systat Software, Inc, Richmond, California). When effects were significant, a Tukey's a posteriori multiple range test was used for pairwise comparisons. Percent data were arcsine transformed to satisfy the ANOVA assumption of normality. A probability level of $P < 0.05$ was considered statistically significant.

Results

Spawning

Milt collected from males before and after ice-breakup was highly motile when activated with NCFW. Ovulated eggs adhered strongly to glass and polypropylene surfaces but the adhesiveness was eliminated by the tannic acid treatment. There were 1,077 eggs/g (egg mass) and a mean of 8,349 eggs per female (226 ± 7 eggs/g body weight). Fertilization success ranged from 69-99%, and development to hatching ranged from 60-86%, at 15°C, for both study years. Hatching was completed by 11 d post-fertilization and larvae were approximately 5 mm at hatch. The fertilized eggs that were disinfected with 50 ppm P.V.P. iodine became stained, crenated and ceased developing.

Hatch experiment

For all temperatures, embryos failed to hatch at 20 and 30 ppt salinity. No significant differences were found between the percentage of larvae that hatched at 10 and 15°C in NCFW (0 ppt) and 10 ppt salinity. Hatching at 5 and 20°C was variable at both 0 and 10 ppt salinity (Table 1). Hatching was complete after 32, 23, 11, and 9 d at 5, 10, 15, and 20°C, respectively.

Effect of green water on growth

Mean survival in GW (87 ± 5.1%) was significantly greater ($p=0.002$) than in clear water (17 ± 4.6%). The mean length of larvae in GW (11.1 ± 0.1 mm) was significantly greater ($p=0.0001$) than for those in clear water (8.2 ± 0.09 mm; Fig. 1).

Effect of light intensity on growth

There was no significant difference between larval survival ($p=0.535$) in low (50 ± 3.0%) and high (54 ± 5.5%) intensity light. The mean length of larvae reared in low intensity light (13.0 ± 0.23 mm) was similar to larvae reared in high intensity light ($p=0.390$; 12.7 ± 0.25 mm).

Effects of temperature and salinity on growth

In the first temperature/salinity experiment, larval survival was similar between rearing temperatures ($p=0.925$; 80.6 ± 3.0% and 82.2 ± 2.7% at 15 and 20°C, respectively) and among salinity levels ($p=0.680$; 80.8 ± 2.3%, 80.0 ± 4.1%, and 83.3 ± 4.0% at 2, 10, and 20 ppt, respectively). As there was no interaction between salinity and temperature, pairwise comparisons were made between temperatures for all salinities and among salinities for both temperatures, for both experiments 1 and 2. The mean length of larvae reared at 20°C (11.4 ± 0.22 mm) was significantly greater ($p=0.039$) than for those reared at 15°C (11.0 ± 0.19 mm). Larvae were significantly longer ($p=0.003$) at 2 ppt (11.9 ± 0.17 mm) when compared to larvae reared at 10 ppt (11.0 ± 0.06 mm) or 20 ppt ($p<0.0001$; 10.6 ± 0.17 mm). Larvae reared at 10 ppt were also significantly longer ($p=0.018$) than larvae reared at 20 ppt (Fig. 2). Results from the second experiment (15 and 20°C at 2, 5 and 8 ppt), were similar to those obtained in the first. Larval survival was similar between rearing temperatures ($p=0.843$; 52.9 ± 2.6% and 52.2 ± 2.1% at 15 and 20°C, respectively) and among salinity levels ($p=0.181$; 48.3 ± 3.0%, 56.3 ± 2.7%, and 53.0 ± 1.8% at 2, 5, and 8 ppt, respectively). Larval length at 20°C (11.7 ± 0.15 mm) was significantly greater ($p=0.0002$) than larval length at 15°C (11.3 ± 0.09 mm). Larval length was inversely proportional to salinity level as larvae reared at 2 ppt salinity (11.9 ± 0.17 mm) were significantly longer ($p=0.004$) than larvae reared at 5 ppt (11.5 ± 0.10 mm) or 8 ppt ($p<0.001$; 11.1 ± 0.07 mm). Larvae reared at 5 ppt were also significantly longer ($p=0.006$) than larvae reared at 8 ppt salinity (Fig. 3).

Discussion

The eggs and larvae of teleost fish are extremely sensitive to environmental stressors (Swanson et al. 1996). Two of the most important variables affecting survival and growth of marine finfish larvae in culture are temperature and salinity (Hart et al. 1996). Optimizing these parameters can improve growth and survival rates and reduce the labor-intensive, larval-rearing period of production (Hart et al. 1996). Salinity and temperature have been reported to influence hatch rate and growth and survival of larvae of other anadromous species including striped bass *Morone saxatilis* and American shad *Alosa sapidissima* (Limburg and Ross 1995; Winger and Lasier 1994). Rainbow smelt eggs hatch over a wide temperature range and hatch can be delayed more than 21 d by decreasing incubation temperature (McKenzie 1964). The natural, longitudinal variation in spawning and slow hatching rate at low temperatures offers culturists a protracted period for spawning that spans more than 6 months. This extended time frame is important for pond culture of smelt in the northeast U.S., since the time of ice breakup and favorable temperatures for plankton blooms varies significantly among years.

The growth, but not survival, of actively feeding larvae was significantly influenced by temperature, and development through metamorphosis was complete at both temperatures tested in this study. Since a very limited temperature range was examined in these studies, no conclusions can be drawn as to the optimal rearing temperature for smelt. The high survival of larvae at 20 °C was somewhat surprising as smelt are considered a coldwater fish with a temperature preference of 6 - 14 °C and have the capacity for antifreeze protein production (Duman and deVries 1974; Klein-MacPhee 2002). For extensive culture in the northeast U.S., however, it will be important to determine the lethal maximum temperature of this species since bottom temperatures in earthen ponds can exceed 24 °C during the summer (J. Whalen, Harmon Brook Farm, personal communication).

The development of the osmoregulatory mechanisms required for saltwater existence is requisite for anadromous fish before entry into the marine environment. Premature exposure of eggs or larvae to high salinity water results in severe dehydration and usually death. In the present study, exposure of smelt eggs to salinity levels of 20 or 30 ppt was lethal, results consistent with those reported for striped bass (Winger and Lasier 1994). Survival of larval smelt was uniformly high (> 78%) at all salinity levels tested but growth was inversely proportional to salt concentration. In other species such as striped bass, (Otwell and Merriner 1975), weakfish *Cynoscion regalis* (Lankford and Targett 1994) and black sea bass *Centropristis striata* (Berlinsky et al. 2000) growth rates were improved by optimizing salinity concentrations. Survival of smelt at salinities greater than 10 ppt greatly facilitates intensive culture of this species, as the marine rotifers *B. plicatilis* and *Artemia* nauplii require this salinity level for optimal growth and survival (Hoff and Snell 1987).

Over the past century, smelt have been introduced into bodies of water by transferring eggs adhered to rocks or burlap. One problem associated with this practice is the high incidence of fungal outbreaks (McKenzie 1964; Akielaszek et al. 1985). In previous experiments, highly variable hatching rates were achieved with adhesive eggs (0 - 80%) and fungal outbreaks were encountered (Akielaszek et al. 1985). Methods to reduce or eliminate egg adhesiveness have been developed for white bass *M. chrysops* and white sturgeon *Acipenser transmontanus* (Kowtal et al. 1986; Rottman et al. 1988). In the present study, high fertilization and hatching rates were attained when egg adhesiveness was eliminated with a tannic acid solution and no fungal infections were encountered. Disinfecting smelt eggs with an iodine solution, however, proved lethal at a concentration half that recommended for salmonid eggs. At least one iodine preparation (Wescodyne™, 5 µg/L) has been used successfully to eradicate fungal infections on smelt eggs (Akielaszek et al. 1985), but it may be prudent to determine the efficacy of other agents such as formalin, ozone and glutaraldehyde.

Most marine fish larvae are visual feeders so the light intensity and turbidity of the water influences their ability to detect prey (Blaxter 1986). Inert particle-induced turbidity has been shown to

enhance larval feeding performance in walleye *Sander vitreus* (Bristow and Summerfelt 1994), but feeding performance was unaffected by turbidity in southern flounder *Paralichthys lethostigma* (Daniels et al. 1996) and decreased in striped bass (Breitburg 1988). Miner and Stein (1993) suggested that an interaction between surface light intensity and turbidity might account for the discrepancy among these reports. At high surface light intensity, prey detection is enhanced with increasing turbidity while at low surface light intensity, below the visual threshold, turbidity reduces prey detection.

Algal cell-induced turbidity (green water) stimulates feeding behavior and improves visibility of prey items for larvae. Furthermore, algae offered as green water provides an important source of micronutrients (Naas et al. 1992; Hjelmeland et al. 1988). In the present study, larval survival was greatly improved when the culture water was supplemented with micro algae but it was unaffected by changes in light intensity. Presumably, the light intensities used here were above the threshold necessary for prey detection. The beneficial effects of green water in this study are in agreement with reports of other marine species including cod *Gadus morhua* (Van der Meeren 1991) and halibut *Hippoglossus hippoglossus* (Naas et al. 1992).

In summary, smelt eggs hatched over a wide temperature range in low salinity water without evidence of fungal infection. Feeding regimes established for marine species using a sequence of enriched rotifers, *Artemia* nauplii and a commercial-weaning diet proved effective for growth of smelt larvae through metamorphosis. Survival of feeding larvae was significantly improved by the addition of green water but was unaffected by the temperatures or salinity levels tested in these studies. The commercial diet used in this study (Biokyowa) is presently not available in the United States. In subsequent studies, rainbow smelt larvae also accepted other commercial diets (e.g. Lansy) and trials examining their suitability are currently underway.

Acknowledgments

We thank Brian Smith and Sherri Paterson (New Hampshire Fish and Game Department), John Whalen (Harmon Brook Farm, Caanan Maine) for assistance with broodstock collection and Manya Hult (UNH) for assistance with culture experiments. This work was funded by the Northeastern Regional Aquaculture Center (NRAC Project No. 02-14). This is Scientific Contribution Number 2209 from the New Hampshire Agricultural Experiment Station

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Table 1. Effect of salinity and temperature on rainbow smelt egg hatching[1]

Salinity (ppt)	Temperature (°C)	Hatch (%)
0	5	7 ± 14[a]
10	5	47 ± 7[b]
0	10	84 ± 7[c]
10	10	85 ± 8[c]
0	15	74 ± 4[c]
10	15	82 ± 3[c]
0	20	42 ± 10[b]
10	20	17 ± 4[a]

[1]Data followed by the same letter superscript indicates no significant difference in hatch (%) between salinities and among temperatures.

No larvae hatched at salinities of 20 and 30 ppt

Figure Legends

Figure 1. Effect of greenwater on growth and survival of rainbow smelt larvae. The micro algae *Nannochloropsis occulata* (200,000 microalgal cells/mL) was added to create the greenwater treatment. Different letters indicate a significant difference between survival or length between bars in the same panel.

Figure 2. Effect of water temperature (15 and 20ºC) and salinity (2, 10, and 20 ppt) on growth (length) of rainbow smelt larvae. Different letters indicate significant differences in length between temperatures and among salinity levels in the same panel.

Figure 3. Effect of water temperature (15 and 20ºC) and salinity (2, 5, and 8 ppt) on growth (length) of rainbow smelt larvae. Different letters indicate significant differences in length between temperatures and among salinity levels in the same panel.

Fig. 1

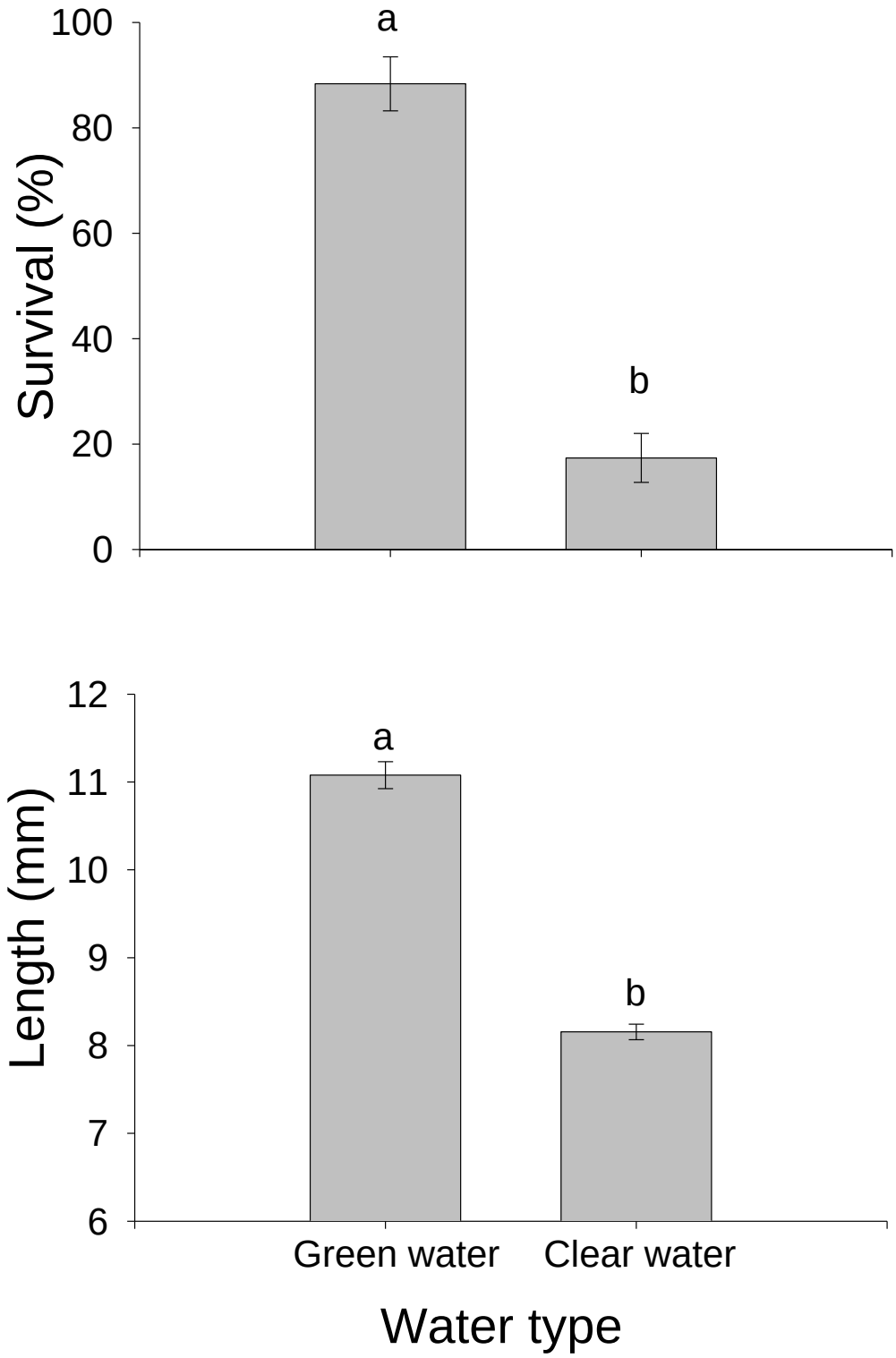
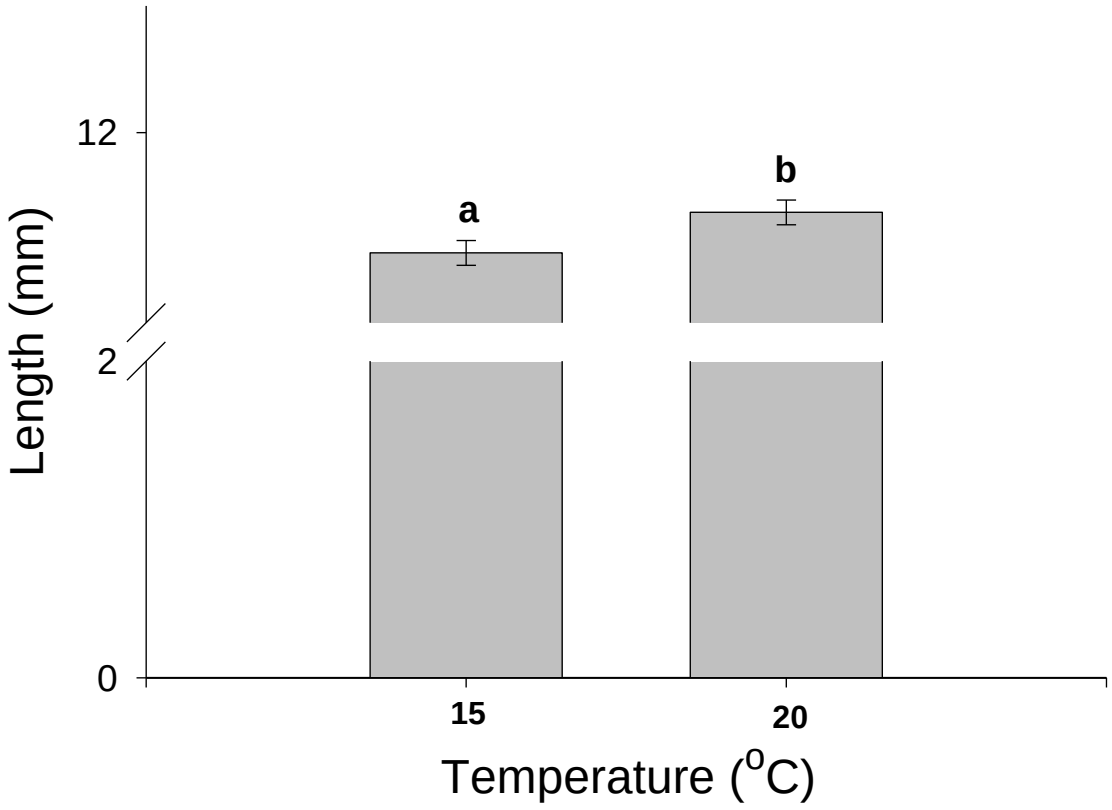
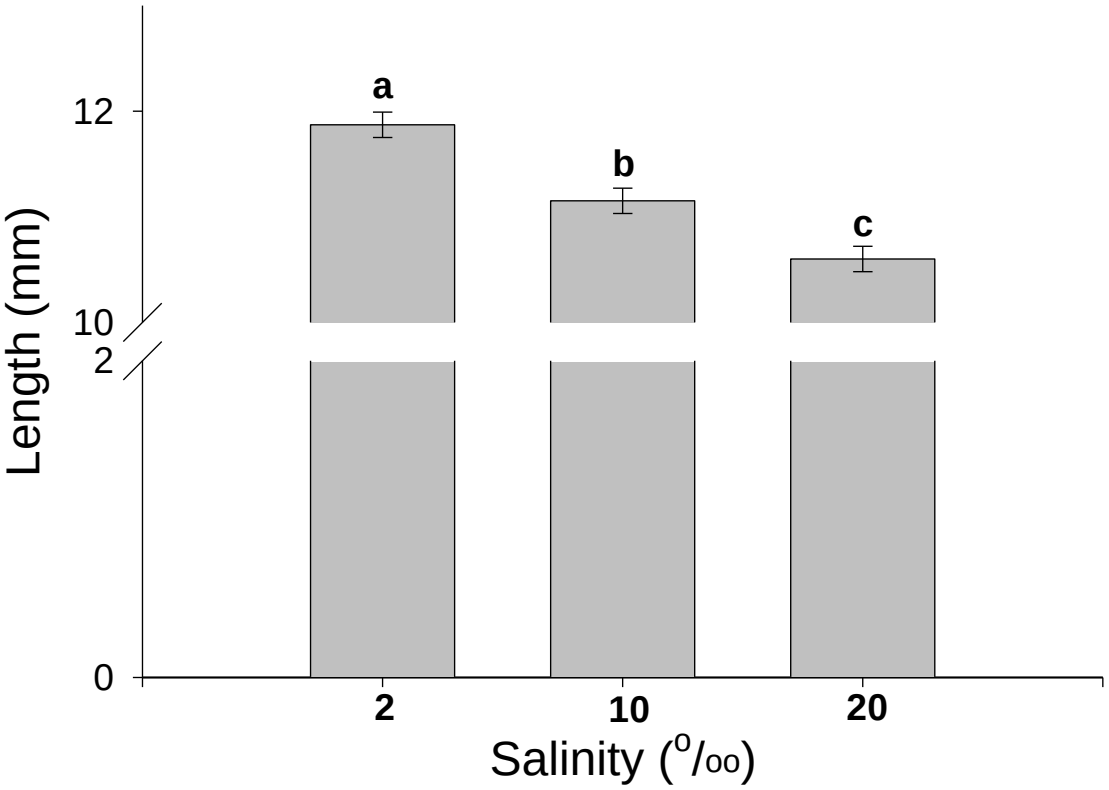
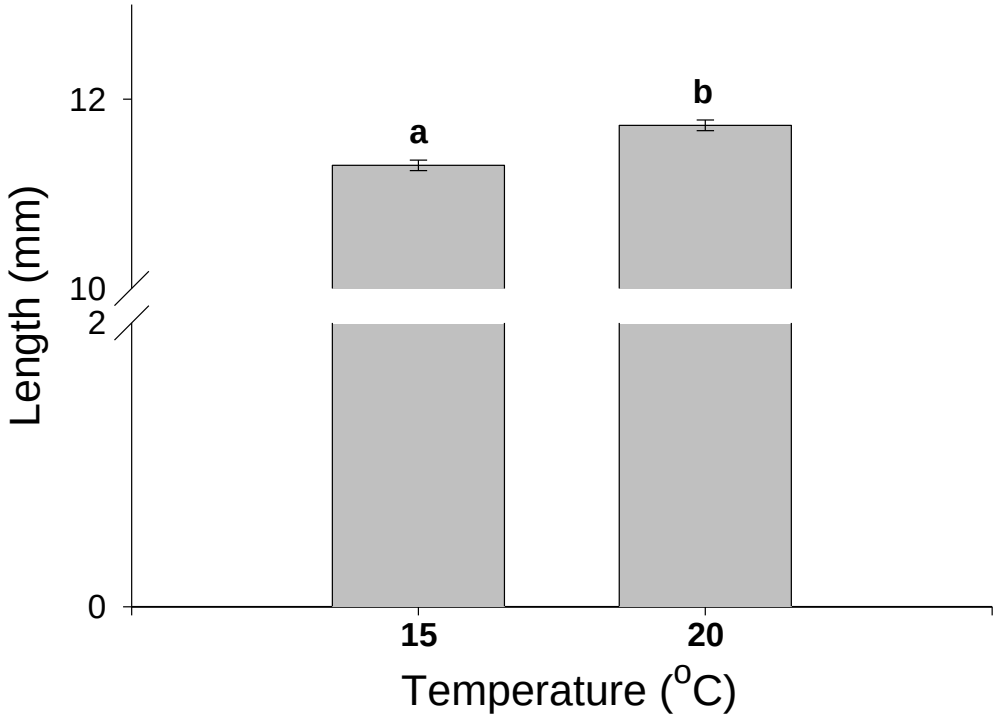
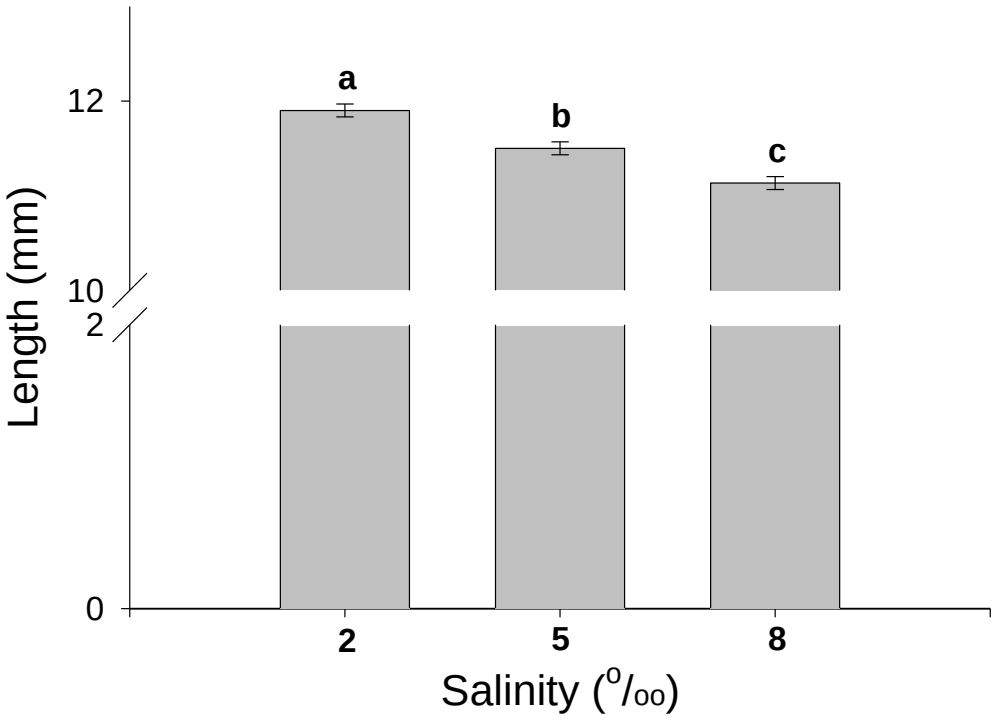

100
80
60
40
20
0
Survival (%)
a
b
12
11
10
9
8
7
6
Length (mm)
a
b
Green water
Clear water
Water type

Fig. 2


12
2
0
Length (mm)
a
b
15
20
Temperature (°C)


12
10
2
0
Length (mm)
a
b
c
2
10
20
Salinity (°/oo)


12
10
2
0
Length (mm)
a
b
15
20
Temperature (°C)


12
10
2
0
Length (mm)
a
b
c
2
5
8
Salinity (°/oo)

Cryogenic and refrigerated storage of rainbow smelt (Osmerus mordax) spermatozoa

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Key words: Cryopreservation, Spermatozoa, Rainbow Smelt

Abstract

The present study describes new methods for refrigerated and cryogenic storage of rainbow smelt (Osmerus mordax) sperm. The effects of extender composition, cryoprotectant (DMSO and methanol), and freezing rate on post-thaw sperm motility were examined. The fertilization capacity of fresh and post-thaw sperm were compared. The highest post-thaw motility (75 ± 1.73) was obtained when semen was diluted with a 0.6-M sucrose solution supplemented with 10% DMSO and 1.5% BSA and frozen at a rate of –20 C/min. Post-thaw motility was not different from that of fresh sperm and did not differ after 90-day storage. Sperm frozen at –30 C/min or with methanol had significantly lower post-thaw motility than sperm frozen at –20 C/min or with DMSO. No differences in fertilization rate or embryo survival to the eyed stage were found between fresh and post-thaw sperm. Refrigerated sperm diluted 1:3 with 0.6-M sucrose remained motile after 30 days.

1. Introduction

The development of sperm cryopreservation has been reported for several anadromous, freshwater and marine fish species (Scott and Baynes, 1980; Gwo, 2000; Lahnsteiner et al. 2002). Cryopreserved sperm may enhance breeding programs by maintaining genetic diversity and variability in domesticated populations, synchronizing gamete availability, limiting broodstock maintenance, aid in the transport of semen, reduce the risk of disease transmission, and preserve gametes from endangered species (Kerby, 1983; Cloud et al., 1990;). The developing methods for salmonid, freshwater, and marine fish sperm cryopreservation have been useful in aquaculture, and may advance the culture of rainbow smelt (Osmerus mordax).

Anadromous rainbow smelt are commercially important finfish in New England, and are one the most widespread species (Akielaszek et al., 1985; Halliwell et al., 2001) found from Labrador to New Jersey (Murawski and Cole, 1978). Smelt are in great demand as a baitfish

(Akielaszek et al., 1985) because they are the preferred prey of Atlantic salmon Salmo salar (Sayers et al. 1989; Pientka and Parrish, 2002). Natural populations are in decline throughout New England due to the construction of dams and reduction in water quality caused by sewage overflow and nutrient loading (Chase and Childs, 2001). Consequently, there is high interest in smelt culture, especially in Maine (Akielaszek et al. 1985).

Several methods have been described for sperm cryopreservation of anadromous species such as rainbow trout Oncorhynchus mykiss (Walbaum) (Lahnsteiner et al. 2002) and striped bass Morone saxatilis (Jenkins-Keeran and Woods, 2002), freshwater species such as yellow perch Perca flavescens (Ciereszko et al. 1993), carp Cyprinus carpio L. (Kurokura et al. 1984; Lubzens et al. 1997), and zebrafish Brachydanio rerio (Harvey et al. 1982), and various marine species (Suquet et al. 2000). There is no data available on rainbow smelt however. Rainbow smelt, turbot Scophthalmus maximus, winter flounder Pseudopleuronectes americanus, plaice Pleuronectes platessa, and zebra fish produce very small volumes of semen that are only available during short time periods. Sperm cryopreservation from such species may therefore extend the spawning period of captive broodstock.

Several factors have been shown to influence the success of cryopreservation including the extender composition, freezing rate, and cryoprotectant concentration (Tiersch, 2000; Fabbrocini, 2000; Sansone et al., 2002). The purpose of this study was to examine the importance of these factors on the survival of rainbow smelt spermatozoa during short and long-term preservation, and to retain high motility and fertilization rates with frozen spermatozoa.

2. Methods

2.1. Fish Collection

Spawning rainbow smelt (18-23 cm length) from Houston Brook, Maine, were collected in April 2003. Fish were transported by truck in an aerated cooler containing water from the area of collection to the University of New Hampshire. Fish were then held in the aerated cooler in a 10 C cold-room with a 0 hr light:24 hr dark photoperiod.

2.2. Gamete Collection

To avoid urine and fecal contamination, fish bladders were cleared by abdominal pressure and the cloaca dried and cleaned with paper towels. Milt was obtained from 4 males by abdominal massage, collected in 1-ml pipet tips, and pooled in 1.5-ml polypropylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA), which were then stored on ice. For fertility trials, smelt eggs were expressed by slight abdominal pressure into a polystyrene weigh boat and handled as detailed below. Fertilization of eggs was conducted 2 days after sperm collection.

2.3. Assessing smelt sperm density and motility

Sperm cell density was determined by diluting (1:200) pooled samples of semen with distilled water and counting cells using an improved Neubauer hemacytometer (West Germany) at 400X with a compound microscope (Olympus CH-2, Melville, NY). The osmolality of smelt seminal plasma was determined after centrifugation (20,000 x g, 10 min) with an Advanced^{TM} 3D3 Osmometer (Advanced Instruments, INC., Norwood, Massachusetts). Spermatozoa were activated for motility estimates by adding distilled water (1:20) and viewed with a hemacytometer as described above. At least 100 cells were examined in less than 2 min.

2.4. Frozen Storage of Sperm

Smelt sperm was frozen using one of three extenders (Table 1) previously used to freeze rainbow trout spermatozoa, two cryoprotectants (dimethyl sulfoxide [DMSO] and methanol, 10%) and two freezing rates (-20 C and –30 C/min. Extenders were made approximately 1 day prior to use. Extenders were mixed and the pH adjusted to 7.8 with HCL or NaOH. Bovine serum albumin (BSA) was added to all extenders as the non-permeating cryoprotectant (Table 1). All solutions and instruments used in the freezing process were chilled (3 C) prior to use. Extenders were mixed with cryoprotectant in 100 x 15mm sterile, polystyrene petri dishes held on ice before being mixed with semen. Diluted semen was aspirated in 3-ml syringes fitted with 20 gauge, 1.5 inch needles and slowly transferred into 0.25-ml cryo-straws (Instruments de Médecine Vétéinaire IMV, L'Aigle, France). Cryo-straws were capped with a sealing powder (Instruments de Médecine Vétéinaire IMV, L'Aigle, France) and held on ice until 8-10 straws were filled. No additional equilibration time was allotted beyond that required to fill the cryo-straws. Cryo-straws containing diluted semen samples were frozen using a Planer Biomed 10-16 programmable freezer (United Kingdom). Once samples reached –150 C, they were immediately plunged into liquid nitrogen. At 1 day post-freezing (DPF), the samples were thawed in 20 C distilled water for 30 sec, the cryo-straws dried and contents emptied into sterile petri dishes at room temperature (20 C). Sperm were activated for motility estimates as described above. At least 100 cells were examined in less than 2 minutes. Samples frozen in the extender that produced the highest 1-day, post-thawed motility were used for fertility trials and were examined again at 30 and 90 DPF.

2.5. Fertilization trials

Freely flowing, ovulated eggs were examined with a dissecting microscope at 25X magnification prior to fertilization trials for confirmation of normal cytological appearance. To ensure that each treatment had approximately the same spermatozoa:egg ratio, 62.5 µl fresh and 250 µl post-thaw sperm were placed directly on 1.0 g eggs (1.4×10^3 eggs/g, 5.8×10^5 spermatozoa/egg) in polystyrene weigh boats. Eggs and sperm were activated with 25 µl filtered (1 µm) non-chlorinated fresh water (NCFW) and swirled for approximately 3 min. Triplicate samples of each treatment were examined. Fertilized eggs were transferred into 220-ml plastic cups containing 150 mg/ml tannic acid and mixed for 10 min. The solution was decanted, and fresh NCFW added. Eggs were then incubated at 15 C with gentle aeration. Due to smelt mortality prior to milt collection, individual comparisons were not made. Rather, frozen milt from a pooled sample of fish was compared with a pool of fresh milt from 4 individuals. Following 24 hrs incubation, fertilization success was determined by examining a minimum of 100 eggs and enumerating those undergoing cleavage. Percent survival to the eyed stage was monitored after 8 days post-insemination.

2.6. Refrigerated Storage

Triplicate samples of smelt sperm were stored neat or diluted 1:1, 1:3, 1:5, 1:10 in extender 3. The sperm samples were stored in labeled 0.5-ml polypropylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) and were refrigerated at 3 C. At 5-day intervals, 1 µl of each sample was removed by pipet, activated, and examined for motility.

2.7. Statistical Analysis

Effects of predictor variables (storage time, extender, cryoprotectant, and freezing rate) on percentage of spermatozoa motility and percentage of eggs fertilized from fresh and cryopreserved sperm were analyzed using full-factorial multi-way ANOVA. When effects were

significant, a Tukey a posteriori multiple range test was used for pairwise comparisons. Percent data were arcsine transformed to improve the analysis of variance assumption of normality. Data are expressed as mean ± SE.

3. Results

3.1. Sperm density and osmolality

Spermatozoa not activated by any of the extenders were examined and osmolality of smelt seminal plasma was 318 mOsm. The pooled, neat sperm contained 8.1 x 10^8 cell ml^{-1} ± 6.36.

3.2. Frozen storage of sperm

The average percent of motile sperm obtained from freshly collected, neat semen was 82 ± 2.68% (Table 2). Differences in sperm motility were found between cryoprotectants ($p < 0.001$) and freezing rates ($p < 0.001$). Higher post-thaw motility was achieved using DMSO and a freezing gradient of –20 C/min (Table 2). Two factor interactions were found between cryoprotectant and extenders ($p < 0.001$) and cryoprotectant and freezing rate ($p < 0.001$). Sperm frozen with DMSO and extender 3 at –30 C/min had higher percent post-thaw motility (46 ± 2.52%) than sperm frozen with extenders 1 and 2 (24 ± 0.1% and 30 ± 2.40% respectively; Table 2). Motility of sperm frozen with extender 3 and DMSO at –20 C/min was approximately 3 times greater (75 ± 1.73%) than that of sperm frozen with methanol at –30 C/min.

The highest motility 1 DPF (75 ± 1.73%) was attained when a freezing gradient of –20 C/min was used with extender 3 and DMSO, however was not different from sperm frozen with extenders 1 or 2 (Table 2). Only sperm frozen in extender 3 supplemented with DMSO retained motility similar to that of fresh spermatozoa motility (Table 2). , Post-thaw motility of sperm frozen with extender 3 after 1 DPF was not different than that after 30 and 90 DPF (74 ± 1.86 and 73 ± 2.08 respectively).

3.3. Fertilization trials

No difference in the fertilization potential between fresh and post-thaw frozen spermatozoa was found, nor was there difference in embryo survival to the eyed stage 8 days after incubation between frozen and fresh spermatozoa (Table 3).

3.4. Refrigerated storage

The motility of neat and diluted rainbow smelt sperm, refrigerated at 3°C is shown in Table 4. The initial motility of fresh, neat sperm (82 ± 1.20%) was not retained after 5 days (72 ± 1.15%), and motility declined rapidly afterwards. Neat spermatozoa were immotile after 10 days. The greatest motility after 10 days (70 ± 3.38%) and most sustained (30 days) motility (5 ± 1.45%) was found with a 1:3 dilution. Sperm diluted 1:1, 1:5, and 1:10 retained lower motility with higher variation after 10 days.

4. Discussion

In this study, the efficacy of 3 extenders, two cryoproctectants, and two freezing rates previously used to cryopreserve sperm from rainbow trout were compared. The best results were obtained when sperm was frozen in an extender containing 0.6-M sucrose supplemented with 10% DMSO and 1.5% BSA. The use of this sucrose solution supplemented with DMSO has been previously suggested in order to maximize fertilization rates of salmonids (Holtz, 1993). Our results corroborate those of Ciereszko and Dabrowski (1996) and Conget et al. (1996) who used a similar extender to cryopreserve rainbow trout Oncorhynchus mykiss sperm. An aqueous 0.6 M sucrose solution with DMSO has previously produced consistent post-thaw fertilization rates when compared with fresh spermatozoa (Holtz et al., 1991; Conget et al. 1996). An extender including sucrose (Mounib, 1978) has also been used successfully on several marine species, including seabass Dicentrarchus labrax (Fauvel et al., 1998) and turbot (Dreanno et al.,

1997; Chereguini et al., 2003). Various other extenders containing saccharides have been developed for anadromous and fresh water species (Wheeler and Thorgaard, 1991; Ciereszko et al., 1993; Lahnsteiner et al., 2002). Sugars, such as sucrose, in sperm extenders, act to stabilize liposomal membranes during freezing (Quinn, 1985; Chereguini et al., 2003), promote cell dehydration (Gwo, 2000), and may act synergistically with DMSO as a cryoprotectant (Gwo, 1994).

DMSO has been a widely used cryoprotectant for many fish species (Scott and Baynes, 1980; Suquet et al., 2000). A sucrose-based extender used with DMSO retained higher post-thaw motility of Atlantic salmon Salmo salar (Mounib, 1978), winter flounder Pseudopleuronectes americanus (Rideout et al. 2003) and turbot (Chereguini et al. 2003) spermatozoa. In the present study, while there was no difference in post-thaw motility between extenders when DMSO was used with a –20 C/min freezing rate only the 0.6-M sucrose extender retained motility similar to that of fresh spermatozoa. The simplicity of this extender facilitates sperm cryopreservtion (Ciereszko and Dabrowski, 1996).

Various cryoprotectants (glycerol, DMSO, methanol) have been widely used, separately or together, for sperm cryopreservation (Wheeler and Thorgaard, 1991; Lahnsteiner et al. 1997; Suquet et al. 2000). While glycerol has been used successfully to preserve Arctic charr Salvelinus alpinus L. sperm (Piironen, 1993), it has been less successful than DMSO in preserving marine fish sperm (Suquet et al. 2000), such as black grouper Epinephelus malabaricus, Pacific herring Clupea pallasi, and Atlantic cod Gadus morhua (Gwo, 1993; Pillai et al., 1994; DeGraaf and Berlinsky, in prep.). This is possibly due to deleterious osmotic and toxic effects of glycerol (Gwo, 2000). The success attained using DMSO with various fish species has been attributed to its fast penetration rate and interaction with membrane

phospholipids (Scott and Baynes, 1980; Baynes and Scott, 1987; Suquet et al. 2000). DMSO was suitable in retaining motility of frozen grayling Thymallus thymallus (Lahnsteiner et al. 1992), yellow perch (Ciereszko et al., 1993) yellowfin seabream Acanthopagrus latus (Gwo, 1994) and Japanese eel Anguilla japonica (Tanaka et al. 2002). Methanol, which may be the most effective cryoprotectant, and applicable for deep freezing sperm of all salmonids studied (Lahnsteiner et al. 2002), has not been shown to be successful for the storage of marine fish spermatozoa (Dreanno et al. 1997; Suquet et al. 2000). While methanol and DMSO caused the smallest reduction in refrigerated sperm motility of channel catfish Ictalurus punctatus, the highest motility was retained in samples frozen with 5 and 10% methanol (Tiersch et al., 1994). Preliminary experiments showed that both DMSO and glycerol were effective cryoprotectants with zebra fish sperm (Harvey et al. 1982). Methanol however, was superior by preventing tail-to-tail agglutination. In the present study, we directly compared DMSO and methanol to determine their efficacies as cryoprotecants for rainbow smelt spermatozoa. Our results indicated that DMSO was superior to methanol in retaining higher post-thaw motility. These results are consistent with those studies using DMSO to freeze various salmonid and marine fish spermatozoa (Baynes and Scott, 1987; Gwo, 1993; Suquet et al. 2000).

An ideal cooling rate prevents the formation of intracellular ice crystals (Chao and Liao, 2001). Various freezing rates and procedures have been used for preserving fish spermatozoa. Sperm has been pelleted onto dry ice, held in liquid nitrogen vapor, or frozen using a programmable freezing (Rana, 1995; Conget et al., 1996; Lahnsteiner et al., 2002). Our results indicated that a –20 C/min freezing rate was superior to one of –30 C/min. Prolonged exposure to DMSO (1-60 min) has been shown to have a negative effect on sperm fertility (Stoss and Holtz, 1983). A programmable freezer allows a short-term exposure of spermatozoa to a

cryoprotectant like DMSO, which can be toxic (Conget et al. 1996; Suquet et al. 2000). Controlled-rate freezers allow for the reproducibility of cooling regimes during cryogenic studies; plastic straws transfer heat rapidly and minimize intracellular ice damage (Rana, 1995).

Straw size may affect the success of sperm cryopreservation in species that produce low semen volumes (≤ 1-ml) like plaice and zebra fish (Pullin, 1972; Harvey et al. 1982). For example, the mean fertilization rate for yellowtail flounder sperm frozen in 1.7-ml straws was lower than that for sperm frozen in 0.25-ml straws, and was significantly lower than fresh sperm (Richardson et al. 1999). Likewise, diluted turbot spermatozoa frozen in 0.20-ml straws retained between 60 to 80% motility upon thawing (Dreanno et al. 1997). While hatching rate with fresh and cryopreserved turbot sperm was not different using various containers (0.5-ml straws and 2-ml cryotubes), high numbers of male broodstock (49 individuals) were needed to fill these containers (Chereguini et al. 2003). When various straw sizes were used to freeze rainbow trout, brown trout, and Arctic charr sperm, fertilization rates were higher with sperm frozen in 0.5-ml straws than in 5-ml straws (Lahnsteiner et al. 1997). Baynes and Scott (1987) used 0.25 straws to preserve rainbow trout spermatozoa with approximately 70% fertilization. In another study, because the average volume of zebrafish semen obtained was 0.8-µl, a 10-µl glass capillary tube was used as a storage container for freezing (Harvey et al. 1982). For rainbow smelt, which need to be sacrificed in order to obtain maximum volumes of semen, a smaller straw size (0.25-ml) was ideal.

Procedures for refrigerated storage of milt have been developed for several teleost species including striped bass Morone saxatilis (Jenkins-Keeran et al. 2001; Jenkins-Keeran and Woods, 2002), channel catfish (Christensen and Tiersch, 1996), and several salmonids (Büyükhatipoglu and Holtz, 1978; Scott and Baynes, 1980; Stoss and Holtz, 1983). Undiluted rainbow trout

sperm, supplemented with penicillin and streptomycin and kept at 0 C, retained full fertilizing capacity after 34 days (Stoss and Holtz, 1983). Previous studies have shown that short-term fish sperm preservation can be improved with the addition of oxygen at 3-4 C (Büyükhatipoglu and Holtz, 1978; Stoss and Holtz, 1983), methanol (Christensen and Tiersch, 1996) or glycine (He and Woods, 2003). In the present study, rainbow smelt spermatozoa diluted 1:3 with the 0.6-M sucrose solution retained motility for 30 days at 3 C. Due to the availability of fish, and time of spawning, fertilization rates for refrigerated smelt sperm could not be compared. Additional studies are required to optimize conditions for refrigerated storage of rainbow smelt sperm.

The present study has provided an optimal protocol for storage of rainbow smelt gametes for New England aquaculture. This study also provides a model for sperm cryopreservation from other smelt species including European smelt Osmerus eperlans, California smelt O. thalichthys, and surf smelt Hyopmesus olidus. The effective storage of gametes is also necessary for the establishment of gene banks for endangered wild stocks (Cloud et al. 1990; Conget et al., 1996). The delta smelt Hypomesus transpacificus has experienced declines in California (Kanim and Jacobsen, 1994) due to increased agricultural practices and alteration of water circulation patterns (Nichols et al., 1986; Monroe and Kelly, 1992) and is currently a threatened species (Kanim and Jacobsen, 1994). This study may offer a successful protocol for gamete storage from declining smelt species.

5. Conclusion

In summary, the present study validates a new method of rainbow smelt sperm cryopreservation, while retaining fertilization capacities and embryonic survival similar to that of fresh sperm. Greater short and long-term spermatozoa survival was found when milt was diluted 1:3 with 0.6-M sucrose solution. Refrigerated spermatozoa remained motile for up to 30 days.

Post-thaw motility was greatest using 10% DMSO as a cryoprotectant with a freezing rate of –20 C/min.

Acknowledgements

We thank Matthew Ayer (UNH) and John Whalin (Harmon Brook Farm) for fish collection. We also thank Richard M. DeGraaf (USFS) and William King V (UNH) for insightful comments on this manuscript. This work was funded by the Northeastern Regional Aquaculture Center (NRAC Project No. 02-14) This is Scientific Contribution Number 2208 from the New Hampshire Agricultural Experiment Station .

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Table and figure legends

Table 1. Composition of extenders for rainbow smelt semen. Modified Lahnsteiner's Extender; Lahnsteiner (Lahnsteiner et al. 20002); Holtz, 1993. Bovine serum albumin (1.5%) was added to all extenders as the non-permeating cryoprotectant.

Table 2. Sperm motility obtained for undiluted and cryopreserved smelt sperm frozen with DMSO and methanol 1 day post-freezing. Different letters represent significantly different values within a freezing rate treatment ($p < 0.05$).

Table 3. Average fertilization and embryonic survival to the eyed stage of eggs fertilized with fresh and cryopreserved sperm. Similar letters represent no significant difference in fertilization and survival between fresh and frozen sperm.

Table 4. Refrigerated (3°C) storage of neat and diluted smelt sperm. Data are mean percent motility $\pm$ SE; n = 3. Values with different letters within a column are significantly different ($p < 0.05$).

	Extender		
	1[a]	2[b]	3[c]
NaCl	103	103	–
KCl (mM)	40	40	–
$CaCl_2$ (mM)	1.0	1.0	–
$MgSO_4$ (mM)	0.8	0.8	–
Hepes (mM)	20	20	–
BSA (%)	1.5	1.5	1.5
Sucrose (%)	–	0.5	0.6 (Mol)
pH	7.8	7.8	7.8
Osmolality (mOsm)	324	463	658

Table 1.

Table 2.

Cryoprotectant (10%)	Freeze Rate (°C min^{-1})	Extender	Motility (%)
0	0	Fresh	82 ± 2.68 a
DMSO	-20	1	69 ± 1.20 b
	-20	2	71 ± 1.33 b
	-20	3	75 ± 1.73 ab
Methanol	-20	1	34 ± 2.03 c
	-20	2	42 ± 1.53 c
	-20	3	40 ± 2.73 c
DMSO	-30	1	24 ± 0.10 efg
	-30	2	30 ± 2.40 ef
	-30	3	46 ± 2.52 d
Methanol	-30	1	14 ± 2.03 h
	-30	2	21 ± 1.67 gh
	-30	3	25 ± 2.19 fg

Table 3.

Table 4.

	Days of Storage			
Dilution	5	10	20	30
Neat	72 ± 1.15 a	1.0 ± 0.88 a		
1:1	81 ± 3.38 a	25 ± 3.18 b	3.0 ± 2.08 a	
1:3	82 ± 1.86 a	70 ± 3.38 c	13 ± 2.08 a	5.0 ± 1.45
1:5	47 ± 3.18 b	47 ± 4.33 d		
1:10	40 ± 4.10 b	40 ± 1.45 d	5.0 ± 3.21 a	

	Fertilization (%)	Survival (%)
Fresh	89 ± 3.46 a	71 ± 3.90 b
Post-thaw Frozen	86 ± 1.20 a	75 ± 4.04 b