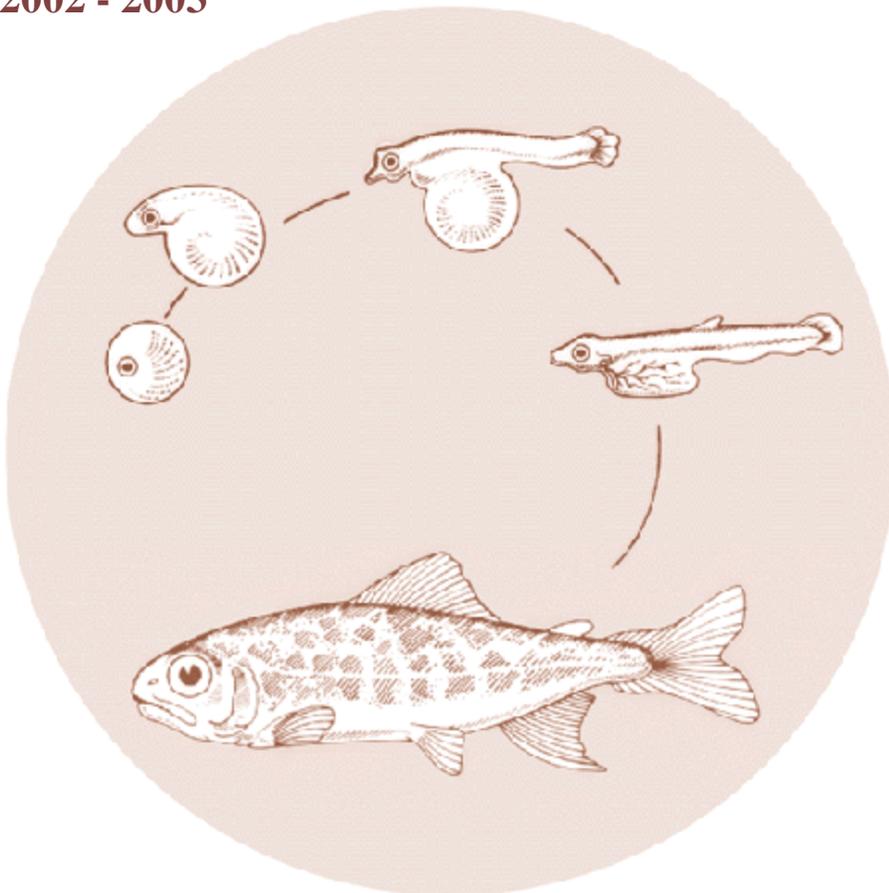


Research on Captive Broodstock Programs for Pacific Salmon

Assessment of Captive Broodstock Technologies

Annual Report
2002 - 2003



This Document should be cited as follows:

Berejikian, Barry, Colin Nash, "Research on Captive Broodstock Programs for Pacific Salmon", Project No. 1993-05600, 207 electronic pages, (BPA Report DOE/BP-00005227-3)

Bonneville Power Administration
P.O. Box 3621
Portland, Oregon 97208

This report was funded by the Bonneville Power Administration (BPA), U.S. Department of Energy, as part of BPA's program to protect, mitigate, and enhance fish and wildlife affected by the development and operation of hydroelectric facilities on the Columbia River and its tributaries. The views in this report are the author's and do not necessarily represent the views of BPA.

**ASSESSMENT OF CAPTIVE BROODSTOCK TECHNOLOGIES
(PROJECT 1993-056-00)**

**ANNUAL REPORT
(PERFORMANCE PERIOD: 1 JUNE, 2002 THROUGH 31 MAY, 2003)**

Prepared by

Barry A. Berejikian

and

Colin Nash

National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Northwest Fisheries Science Center
Resource Enhancement and Utilization Technologies Division
P.O. Box 130
Manchester, Washington 98353

Funded by

US Department of Energy
Bonneville Power Administration
Environment, Fish and Wildlife
P.O. Box 3621
Portland, Oregon 97208-3621

Project No. 1993-056-00
Contract No. 99-AI-17859

July 2003

TABLE OF CONTENTS

ABSTRACT	5
LIST OF AUTHOR AFFILIATIONS	7
OBJECTIVE 1 - IMPROVE NATURAL REPRODUCTIVE PERFORMANCE	8
TASK 1. EVALUATE THE EFFECTS OF EXERCISE-TRAINING ON REPRODUCTIVE PERFORMANCE, AND ASSOCIATED CHARACTERISTICS	8
Introduction	8
Methods and, Materials, and Description of Project Areas	9
Results and Discussion	12
Data Management Activities	15
Summary and Conclusions	15
References	25
OBJECTIVE 1 - IMPROVE NATURAL REPRODUCTIVE PERFORMANCE	26
TASK 2. EVALUATE AND MONITOR THE BREEDING BEHAVIOR AND SUCCESS OF ESA-LISTED CAPTIVELY-REARED CHINOOK SALMON IN IDAHO STREAMS	26
Introduction	26
Materials and Methods, including Description of Study Area	26
Results and Discussion	27
Data Management Activities	27
Summary and Conclusions	27
OBJECTIVE 2 - IMPROVE OLFACTORY IMPRINTING	28
TASK 3. DETERMINE CRITICAL IMPRINTING PERIODS FOR SOCKEYE SALMON	28
Introduction	28
Materials, Methods, and Description of Study Area	29
Results and Discussion	30
Data Management Activities	33
Summary and Conclusions	33
References	39
OBJECTIVE 3 - IMPROVE PHYSIOLOGICAL DEVELOPMENT AND MATURATION	40
TASK 4. THE EFFECTS OF GROWTH RATE ON INCIDENCE OF EARLY MALE MATURITY AND ADULT QUALITY IN SPRING CHINOOK SALMON	40
Introduction	40
Materials, Methods, and Description of Study Area	42
Results and Discussion	44
Data Management Activities	45
Summary and Conclusions	45
References	50
TASK 5. DETERMINE EFFECTS OF GROWTH ON MATURATION TIMING, FECUNDITY, EGG SIZE, AND EGG QUALITY IN COHO SALMON	51
Introduction	51
Materials, Methods, and Description of Study Area	53
Results and Discussion	55

Data Management Activities	59
Summary and Conclusions	59
References	71
TASK 6. MONITORING REPRODUCTIVE DEVELOPMENT IN CAPTIVE BROODSTOCK AND ANADROMOUS HATCHERY STOCKS OF SNAKE RIVER SPRING CHINOOK SALMON DURING THE FRESHWATER PHASE OF ADULT MIGRATION	74
Introduction	74
Materials, Methods, and Description of Study Area	76
Results	79
Data Management Activities	84
Discussion and Conclusions	84
References	91
TASK 7. EFFECTS OF WATER TEMPERATURE DURING THE SEAWATER REARING PHASE ON THE TIMING OF SPAWNING AND EGG QUALITY IN SPRING CHINOOK SALMON.....	135
Introduction	135
Materials, Methods, and Description of Study Area	136
Results and Discussion	137
Data Management Activities	138
Summary and Conclusions	139
References	145
OBJECTIVE 4 - IMPROVE IN-CULTURE SURVIVAL OF JUVENILES: PREVENTION AND CONTROL OF DISEASE.....	147
TASK 8. DEVELOPMENT AND TESTING OF VACCINES AND CHEMOTHERAPEUTANTS TO REDUCE MORTALITY FROM BACTERIAL KIDNEY DISEASE (BKD) IN CHINOOK SALMON.....	147
Introduction	147
Materials, Methods, and Description of Study Area	148
Results and Discussion	150
Data Management Activities	153
Summary and Conclusions	153
References	159
OBJECTIVE 5 - EVALUATE EFFECTS OF INBREEDING AND INBREEDING DEPRESSION.....	161
TASK 9. DETERMINE THE EFFECTS OF CONTROLLED INBREEDING ON SURVIVAL, DEVELOPMENT, AGE STRUCTURE, AND OTHER ASPECTS OF THE LIFE HISTORY OF CHINOOK SALMON	161
Introduction	161
Materials, Methods, and Description of Study Area	162
Results and Discussion	163
Data Management Activities	170
Summary and Conclusions	170
References	179

APPENDIX A.....	i
OBJECTIVE 3 - IMPROVE IN-CULTURE SURVIVAL OF JUVENILES: PREVENTION AND CONTROL OF DISEASE.....	i
FINAL REPORT FOR TASK 10 FROM FY 1999 - 2002. USE OF ANTIBIOTICS AND NEW VACCINES TO REDUCE MORTALITY FROM BACTERIAL KIDNEY DISEASE IN CHINOOK SALMON	i
Introduction	i
Materials, Methods, and Description of Study Area	iii
Results and Discussion	v
Materials, Methods, and Description of Study Area	xiii
Results and Discussion	xvi
Summary and Conclusions	xxiii
References	xxv

ABSTRACT

The success of captive broodstock programs depends on high in-culture survival, appropriate development of the reproductive system, and the behavior and survival of cultured salmon after release, either as adults or juveniles. Continuing captive broodstock research designed to improve technology is being conducted to cover all major life history stages of Pacific salmon.

Current velocity in rearing vessels had little if any effect on reproductive behavior of captively reared steelhead. However, males and females reared in high velocity vessels participated a greater number of spawning events than siblings reared in low velocity tanks. Observations of nesting females and associated males in a natural stream (Hamma Hamma River) were consistent with those observed in a controlled spawning channel. DNA pedigree analyses did not reveal significant differences in the numbers of fry produced by steelhead reared in high and low velocity vessels.

To determine the critical period(s) for imprinting for sockeye salmon, juvenile salmon are being exposed to known odorants at key developmental stages. Subsequently they will be tested for development of long-term memories of these odorants. In 2002-2003, the efficacy of EOG analysis for assessing imprinting was demonstrated and will be applied in these and other behavioral and molecular tools in the current work plan. Results of these experiments will be important to determine the critical periods for imprinting for the offspring of captively-reared fish destined for release into natal rivers or lakes.

By early August, the oocytes of all of Rapid River Hatchery chinook salmon females returning from the ocean had advanced to the tertiary yolk globule stage; whereas, only some of the captively reared Lemhi River females sampled had advanced to this stage, and the degree of advancement was not dependent on rearing temperature. The mean spawning time of captive Lemhi River females was 3-4 weeks after that of the Rapid River fish. Captive Lemhi River females produced smaller and fewer eggs than the Rapid River females; however, relative fecundity was higher than that of the Rapid River fish.

Female coho salmon that ceased or slowed oocyte development in the spring had lower body growth from the previous August onward compared with females that continued oocyte growth. This indicates that growth during the late summer and fall, one year prior to spawning, can determine the decision to mature the following spring. Therefore it is important to maintain the growth of broodstock during the summer/fall period to ensure the continuation of ovary development in the subsequent spring.

A combined whole cell vaccine of Renogen with killed *R. salmoninarum* strain MT239 may be effective in reducing the occurrence of BKD during the period immediately after seawater transfer, but not in yearling seawater-adapted chinook salmon. Control of BKD is likely to require an integrated disease management plan, utilizing three components, namely broodstock segregation, antibiotics, and vaccination. Vaccine results incorporated with antibiotic treatment will be used to work toward an integrated disease

management plan to help to reduce the cycle of BKD transmission in the captive stocks to increase survival safely.

Patterns of estimated survival in one chinook salmon stock (Grovers Creek) were generally consistent with inbreeding depression: progeny of fish that were full siblings (approximate increment in F of 0.25) survived to return at much lower rates than did progeny of fish that were half siblings ($F \sim 0.125$) or unrelated individuals ($F \sim 0$). Growth at sea of Grovers Creek Hatchery stock study fish was lower than that of UWH stock fish. Among the inbreeding groups alone, no clear differences in growth were detectable. However, preliminary results suggest the general pattern of growth was opposite that expected if inbreeding depression reduced growth: the highest growth was in progeny of related parents.

LIST OF AUTHOR AFFILIATIONS

**National Marine Fisheries Service, Northwest Fisheries Science Center, 2725
Montlake Blvd. E., Seattle, WA 98112-2097**

Brian R. Beckman, Andrew Dittman, Brad Gadberry, Jeffrey J. Hard, Donald Larsen,
Cyndy L. Masada, Mark S. Myers, Mark E. Peterson, Linda D. Rhodes, Karl Shearer,
Mark S. Strom, Penny Swanson, Gary Winans,

**National Marine Fisheries Service, Northwest Fisheries Science Center, Manchester
Research Station, P. O. Box 130, Manchester, WA 98353**

Barry A. Berejikian, Lee W. Harrell, William C. McAuley, Cindra K. Rathbone,
Christopher Tatara, Skip Tezak.

**Pacific States Marine Fisheries Commission, 45 SE 82nd Drive, Suite 100,
Gladstone, Oregon 97027**

Rebecca K. Andrews, Jeff Atkins, William T. Fairgrieve, Eric Kummerow, Anita L.
LaRae, Paul Parkins

**University of Washington School of Aquatic and Fishery Sciences, Box 355020,
Seattle, WA 98195-5020**

Briony Campbell, Kathy Cooper, Nicholas Hodges^{2, 3}, Jon Dickey, Kerry A. Naish.

Department of Biological Sciences, University of Idaho, Moscow, Idaho 83844

Graham Young

Idaho Department of Fish and Game, Boise, Idaho 83686

Paul Kline, Dave Venditti

OBJECTIVE 1 - IMPROVE NATURAL REPRODUCTIVE PERFORMANCE

TASK 1. EVALUATE THE EFFECTS OF EXERCISE-TRAINING ON REPRODUCTIVE PERFORMANCE, AND ASSOCIATED CHARACTERISTICS

By

Barry Berejikian, Anita LaRae, Skip Tezak, Jeff Atkins, Eric Kummerow

Introduction

Pacific salmon (*Oncorhynchus* spp.) reared to the adult stage and released for natural spawning exhibit a lower level of reproductive performance than wild salmon. Hormonal processes during maturation (Berejikian et al. 2003) and possibly physical fitness may contribute to reduced reproductive success. Improving natural reproductive performance is critical for the success of captive broodstock programs in which adult-release is a primary reintroduction strategy for maintaining ESA-listed populations.

In the 2000 Federal Columbia River Power System (FCRPS) Biological Opinion, NMFS has identified six populations of steelhead and several salmon populations which have dropped to critically low levels, and continue to decline. Following thorough risk-benefit analyses, captive propagation programs for some or all of these steelhead populations may be required to reduce the risk of extinction, and more programs may be required in the future. Thus, captive propagation programs designed to maintain or rebuild steelhead populations require intensive and rigorous scientific evaluation, much like other project objectives currently underway with chinook and sockeye salmon.

Three captive broodstock programs in the Columbia and Snake River Basins currently, or have in the past, released captively reared adults for natural spawning, rather than artificially spawning those fish and releasing their progeny as juveniles. Previous NMFS studies have evaluated the breeding behavior and reproductive success of captively-reared coho and chinook salmon. Captively-reared coho salmon exhibit inferior competitive ability compared with wild coho salmon, and female mate selection appears to favor wild over captively-reared males (Berejikian et al. 1997, Berejikian et al. 2001a). In chinook salmon, direct comparisons have not been made between captively-reared and wild adults; however, several anomalous behavior patterns have been observed and documented; for example, females frequently abandoned their nests and even after several hours of continuous courtship and numerous male ejaculations, never returned to spawn. Sexually mature males often showed little or no courtship behavior when nearby nest-digging females were present (Berejikian et al. 2001b). In addition to the reproductive behavioral deficiencies in Pacific salmon, the eggs of adult captively-reared chinook salmon spawning in natural streams in Idaho suffered greater mortality than that of artificially spawned cohorts (P. Kline, IDFG, personal communication).

In order to guide future decisions on captive broodstock reintroduction strategies and improve husbandry practices, it is critical to determine whether spawning performance can be improved by modifying rearing protocols, and to understand the rearing environment factors affecting reproductive performance. This work evaluates the effect of one environmental factor, current velocities in the rearing tanks, to exercise the fish and possibly influence physical fitness.

Much literature suggests that exercise positively influences some parameters of salmon health, including fin quality (Joergensen and Jobling 1993), growth rate (Houlihan and Laurent 1987), muscle mass (Barret and McKeown 1988), and swimming stamina (Leon 1986, McDonald et al. 1997). A recent study with captively-reared chinook salmon determined the effects of exercise on reproductive performance (Berejikian et al. 2003). In that study, chinook salmon were exercised at 0.5 to 0.75 body lengths per second current velocity for approximately 5 months during seawater rearing. Exercised and non-exercised fish were subsequently combined into low velocity freshwater vessels for approximately 2 months prior to final maturation. The duration and timing of exposure to a high velocity environment may have been inadequate to affect characteristics that would improve reproductive performance. In females exercise had no effect on the onset of spawning or egg deposition, two primary factors limiting the reproductive success of captively-reared salmon. Thus, in chinook salmon exercise probably provided only a marginal benefit during spawning (see Berejikian et al. 2003 for details).

The present work was designed to determine the effects on steelhead of increased current velocities. Steelhead reproductive characteristics were evaluated at maturity (age-4). Replicate groups of steelhead had been exposed to elevated current velocities for nearly 2.5 years. Evaluations were conducted under controlled experimental conditions and in the natural stream from which the steelhead were collected as embryos.

Methods and, Materials, and Description of Project Areas

In 1998, eyed eggs were hydraulically sampled from eight naturally constructed steelhead redds in the Hamma Hamma River as part of a collaborative stock restoration program. The Hamma Hamma River enters the west side of Hood Canal approximately 15 km north of the town of Hoodport, Washington. Some of the eggs were allocated to an experimental captive rearing program to test the efficacy of the adult release strategy, and the effects of exercise via increased current velocities on adult reproductive performance. The steelhead were raised at Long Live the Kings (LLTK) Hatchery, approximately 10 km south of the Hamma Hamma River. Redd-specific groups of fish were cultured in separate 1.8-m diameter tanks until each fish could be PIT-tagged for individual identification. A total of 296 steelhead were divided equally into four circular rearing tanks (6 m i.d.) in October 1999. Pumps (2.0 HP) and variable speed controllers were installed in two tanks to re-circulate water and create current velocities up to 0.60 m/sec. Current velocities in these two high velocity (HV) tanks were increased to approximately 1 body length/s (BLPS) for 12 hr each day between 22 October 1999 and 27 March 2000,

after which velocities were increased to the same velocity for 23 hr/day. Current velocities were held at less than 0.25 BLPS in the two remaining low velocity (LV) tanks. Fish in all tanks were fed similar rations. Fork length, weight and were measured throughout the rearing period, and maturation rates at age-3 and age-4 were recorded. A total of 84 maturing females and 116 maturing males were injected with yellow (HV treatment) or white (LV treatment) individually numbered anchor tags before being released into the Hamma Hamma River for natural spawning on 28 February 2002. A total of 64 maturing adults (32 males and 32 females) were transported to the NMFS Manchester Research Station (MRS) on 24 February 2002. Each fish was anesthetized, weighed, measured, and an individually numbered Peterson Disk tag (2.5 cm) was attached to each fish for individual identification. Twelve males and 12 females were released into each of two replicate 40-m long by 3-m wide sections of the experimental spawning channel at the MRS. Fish could not move between these two sections. The LV and HV treatments were equally represented in each channel section. The channel had water depths (25 - 35 cm), current velocities (up to 0.5 m/s), gravel size (1 - 10 cm diameter), and temperatures within the documented range of natural spawning habitat for the species. The channel was supplied with well-water (80 l/minute), which was re-circulated at a flow rate of approximately 6,800 l/minute. A chiller unit maintained temperatures between 5.8 and 9.5°C throughout the spawning period. Each side of the channel contained 8 discrete sub-sections (5 m long by 3 m wide), each separated by a 15 cm high water fall. Fish in different sections could not see fish in adjacent upstream or downstream sections, but could readily pass from one to another.

To quantify reproductive behavior, each section of the 16 sub-sections was observed from behind an observation blind for three 10-min periods (scans) each day. During each scan, females were assigned one or more of the following status designations: nest building, redd holding, and courted by male. Females were designated as nest building if they exhibited digging behavior consistent with nest construction (i.e., excavation of gravel from a defined nest depression). Females were designated as redd-holding if they were within 1 m of a redd that they had constructed, and females were designated as courted when the female was over or near (within 1-m) of a redd and at least one male was courting her (i.e., quivering and crossing over). The number of times each fish was observed in each of the categories was expressed as a proportion of the total number of (scans). The frequency of aggressive attacks by an individual female against other females and males was quantified during each scan and. For males, the proportion of scans in which each male was observed to be courting a female (i.e., quivering and crossing over) was also calculated. The frequency of aggressive attacks by an individual male against other males and females was quantified during each scan and the frequency per 10-min scan was calculated.

In addition to scan sampling, reproductive behavior was monitored by underwater and overhead cameras. Four cameras (Watec¹, model 902HS; 00015 lux sensitivity @ F1.4; 26 mm lens) were positioned approximately 3.8 m above the stream channel, so that each camera captured images from 25% of the channel. Video signals were continuously recorded on time-lapse recorders (Gyrr¹, model TLC 2124-GY) at approximately 5 frames/sec between 1700 hrs and 0730 hrs. All night-time spawning events were

recorded and observed upon reviewing the video tapes between 0800 and 1200 hrs the following day. During daylight, three remote underwater cameras were used to record spawning events. When a female appeared to be preparing a new nest for spawning, a camera was positioned nearby, and the video signals were recorded in real time (JVC¹ Super VHS, model HR S-7300-U).

For each spawning event observed either directly or via underwater video recordings, the male that had established the 'dominant' position (next to the female) and those that participated by 'sneaking' into the nest and ejaculating during oviposition (see Foote et al. 1997) were identified. The frequency with which each fish was observed to be either dominant or sneaking over the entire spawning period was tabulated. Combining information from the scan observations and over-night time-lapse video recordings also allowed us to determine each female's onset of spawning, spawning duration, and numbers of nests constructed.

Spawning activities recorded on underwater video tape also provided a continuous temporal profile of behaviors before, during, and after spawning. The frequency of courtship behaviors by the male (such as crossovers, quivers, nudges, and gaping) and nest construction activities by the female (digs and probes) are being analyzed directly from the video tapes. Analog video has been compressed to Mpeg format for analysis using the Observer behavior recording program (Version 4.0). Frequencies of each behavior were tabulated for six consecutive 10-minute intervals prior to spawning and three consecutive 10-minute intervals after spawning (e.g., Berejikian et al. 2000).

Once all spawning had been completed, the adults were removed from the spawning channel. Muscle tissues and internal organs (gonads removed) were collected, sealed, and frozen. Analyses were performed for whole body proximate composition, which quantified the relative proportions of protein, lipid, ash, and moisture content in the body.

Individual fish were prepared for analysis by repeatedly grinding the whole, partially-thawed carcasses in a silent cutter (Hobart Corp¹) until a smooth homogenate was obtained. Approximately 100 g of each wet sample was dried to a constant weight and stored in sealed glass vials at -20°C . Methods described in AOAC (1995) were followed for proximate analysis of samples: moisture by oven drying to constant mass (16 h) at 105°C , protein (N \cdot 6.25) by nitrogen determination using a LECO FP 428 analyzer, crude lipid by Soxhlet extraction with dichloromethane, and total ash by combustion at 550°C for 16 h.

A pedigree analysis was conducted to determine the adult-to-fry reproductive success of individual steelhead released into the spawning channel. The downstream-end of each stream channel section was fitted with a trap in June 2002 to collect fry which had emerged from the gravel and emigrated. Fry traps were checked several times per week and all fry were removed. Samples from a given week were combined and kept separate until all fry had been collected from the traps, and all non-emigrating fry were removed from the stream channel by seining and electro-shocking. One thousand fry were sub-sampled in such a manner to represent the weekly samples proportionately (see Results

and Discussion). Muscle tissue samples were taken from these fry and preserved in 100% non-denatured ethanol.

Genomic DNA were extracted from the adult and fry tissue samples, and subjected to polymerase chain reactions (PCR) to amplify 6 - 20 loci known to exhibit microsatellite polymorphism for steelhead. A fragment analysis was conducted on the PCR products using an Applied Biosystems 310 genetic analyzer to determine the genotypes of every individual for each locus. The genotypes of the fry were compared with those of the adults using the computer program CERVUS to determine the parentage of each fry.

Behavioral response variables were analyzed by a two-way mixed model Analysis of covariance (ANCOVA), with body weight as the covariate. The individual rearing tanks (two tanks per treatment) were nested within current velocity (fixed main effect). Channel side (A and B) was the other main effect (random). To improve homogeneity of variance, two variables, the proportion of observations in which a female was courted by a male and the proportion of eggs deposited by females, were arcsine transformed. Aggression by females towards males was square-root transformation.

The proportion of the fry population produced by each adult was calculated within each of the two channels. A single factor, ANCOVA was performed to test for the effects of current velocity (fixed main effect), body weight (covariate) and rearing tank (nested within current velocity) on the proportion of fry produced.

The behavior of captively-reared steelhead released into the Hamma Hamma River was monitored several days each week from the stream bank, and by underwater video between 28 February and 10 June 2002. Observers walked along both stream banks to locate females that were constructing nests. When a nest-digging female was encountered, an attempt was made to determine its rearing treatment (captively-reared, sea-ranched, or wild) based on presence or absence of an anchor tag, and its color (HV fish received yellow tags and LV fish received white tags). Dominant and sneaker males interacting with the female were also identified to rearing treatment where possible. All females observed were categorized as either nest digging, redd covering, nest guarding, or inactive. Males were categorized as either dominant or sneaker. The water depth and velocity will be measured for each new redd observed in which the type of female constructing the redd was known.

A portion of the eyed embryos in redds constructed by known parents was hydraulically removed after they have reached the eyed stage of development (~230 CTUs). Thus, redd sampling occurred in July and August 2002. The proportion of viable embryos was calculated and compared to previous years in which only wild fish had spawned in the Hamma Hamma River.

Results and Discussion

The results provided are considered to be only preliminary. A discussion of the finalized results will be submitted with the FY 2003 Annual Report.

In late winter and early spring, the majority of the 276 captively-reared steelhead sexually matured. There were no significant differences in the rate of maturity of males or females between the two rearing treatments ($P > 0.05$ for all comparisons). In the high velocity rearing tanks an average (\pm s.d.) of 30.1% (\pm 3.1%) of the population were mature females, 45.6% (\pm 4.2%) were mature males, and 24.3% were non-maturing. In the low velocity rearing tanks an average of 24.4% (\pm 2.9%) were mature females, 48.1% (\pm 2.6%) were mature males, and 27.5% were non-maturing. A total of 268 of the 276 ARG fish (97.1%) survived to age-4, out of which 261 (97.3%) matured. Growth was very similar among the four rearing tanks and between the two treatments (Figure 1). The LV and HV-reared steelhead had similar body composition at the end of the spawning period (Figure 2).

Individual rearing tanks (nested within the main effect of current velocity) had no effect on any of the reproductive behaviors quantified for males and females ($P > 0.05$ in all cases). Tests for interactions between the main effects of current velocity and channel side were also non-significant for all behavioral response variables for males and females ($P > 0.05$ in all cases). Likewise, body size did not have a significant effect on any of the behavioral response variables recorded for males and females (Tables 1 and 2). Males reared under LV conditions were more aggressive towards other males than were HV-reared males, and LV-reared males were observed to be courting females in a greater proportion of scans than LV-reared males (Tables 3 and 4). Egg deposition (calculated as the proportion of a female's estimated fecundity) was significantly greater in channel B (99.8%) than in channel A (97.7%); however, the relative difference was small. The main effects of current velocity and channel side did not have significant effects on any other behavioral response variables (Tables 1 and 2).

A total of 50 individual spawning events were recorded via underwater videography with enough clarity and duration (at least 1 hour of documentation of both pre-spawning courtship and 0.5 hours post-spawning behaviors) to enable detailed analysis of courtship behavior. Recorded spawning events spanned the duration of study period (February to the last week of April 2002). The following paragraphs summarize information gathered on mating combinations and briefly describes nest building and courtship behavior.

Of the 50 spawning events recorded during daylight hours on underwater cameras or by direct observation, 18 involved HV females (7 different females), and 35 involved LV females (9 different females). The number of spawning events recorded for individual females ranged between 0 and 7. The dominant male was identified in 42 of the 50 events. Males reared in low velocity tanks were dominant in 35 of the 42 spawning events in which a courting male was identified, and HV males attained courting status in the remaining 7 spawning events.

The majority of the matings (44%) involved a LV female and a dominant LV male. Twenty-six percent of the matings involved an HV female and a dominant LV male, and 14% involved a LV female and a dominant HV male. None of the observed matings involved an HV female and dominant LV male. Ten of the 50 spawning events captured

on underwater video included a 'sneaker' male that darted into the nest and released milt at the time of egg deposition.

An initial review of the recorded spawning events indicated the following. During the period (<1 hour) leading up to spawning, female steelhead appeared to exhibit fewer overall nest digs than has been reported for chinook and coho salmon. Males exhibited courtship behaviors towards females regardless of whether or not the female was actively constructing a nest. Males quivered alongside the females, but quivering varied substantially with regard to body flexion frequency and proximity to a female. Some quivers were angled away from the female or were at a distance greater than ~ 0.3 m from the female. The frequency of male crossovers increased more dramatically than quivers as the spawning event approached (Berejikian et al., 2001). Males tended to remain alongside the female beyond five minutes post-spawning. In several of the recorded spawning events, males were seen nosing into the nest and eating eggs that had just been deposited.

A total of 15,367 fry emigrated from the stream channel between 10 June and 17 August 2002. By 17 August 2002, the emigration rate had slowed considerably (Figure 3) and the fry remaining in the channel were removed by seining and electro-shocking. Combining fry that emigrated and those remaining (residents) in the channels, a total of 6,134 fry was produced from channel A and 12,103 from channel B. Sub-sampling for parentage assignment was performed at an equal rate of 5.48% of all emigrants from channel A (250 fry) and channel B (593 fry) and residents from channel A (86 fry) and channel B (71 fry). The 5.48% weekly sub-sample rate was applied equally across each of the 10 weekly trap collections.

The proportion of fry (residents and emigrants combined) produced by individual males was unaffected by rearing treatment ($F_{1,19} = 0.240$, $P = 0.630$), tanks nested within treatments ($F_{2,19} = 2.139$, $P = 0.145$), and body size ($F_{1,19} = 1.294$, $P = 0.269$) (Figure 4). Likewise, rearing treatment ($F_{1,19} = 0.018$, $P = 0.893$), tanks nested within treatments ($F_{2,19} = 0.710$, $P = 0.504$), and body size ($F_{1,19} = 0.004$, $P = 0.950$) did not affect the proportion of fry produced by individual females (Figure 4).

Between 1 March and 31 May 2002, a total of 24 females were observed to be actively courted by males in the Hamma Hamma River. Eleven LV females, 6 HV females, and 7 anadromous females (either wild or hatchery-reared) were observed being courted.

The treatment combinations of courting pairs are shown in Table 5. The viability of embryos hydraulically removed from redds that were constructed by captively reared females ($n = 5$) and from redds in which a captively reared male was observed holding over the redd ($n = 4$) averaged greater than 94.1% (Table 6). The embryo viability in those redds is within the range measured for wild fish spawning in the Hamma Hamma River (86% to 98% annual average) between 1998 and 2001 (Berejikian et al. 2002).

Data Management Activities

Data are collected by NOAA and PSMFC researchers onto preformatted data sheets or directly into electronic spreadsheets or text files. Data are entered and summarized on personal computers operated by researchers; primary software used for these procedures includes Microsoft Excel 2000 and Word 2000. Systat 10 is used for statistical analyses. All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report under Materials and Methods. Data analyses are reported in the Results section of the annual report. Data analyses that are incomplete will be included in the subsequent annual report(s).

Summary and Conclusions

In summary, it appears current velocity has little if any effect on the frequencies of male and female reproductive behavior frequencies. However, participation in spawning events was observed to be much greater for LV than HV-reared males and females. Observations of nesting females and associated males in the Hamma Hamma River were consistent with those observed in the stream channel and MRS. Although HV females and males were less frequently observed spawning, DNA pedigree analyses did not reveal any significant differences in the numbers of fry produced by HV and LV-reared steelhead. Final conclusions will be drawn following final data analysis and reporting.

Table 1. Results of the two-way ANCOVA with body size as a covariate to determine the effects of current velocity and channel side on female reproductive behavior. An asterisk indicates significance of probability (P) values at $\alpha = 0.05$.

Class	DV	Current Velocity		Channel		Body Size	
		F	P	F	P	F	P
Nest Construction							
	Nest Building	0.084	0.821	1.846	0.192	0.567	0.228
Aggression							
	To female	0.608	0.578	0.245	0.627	2.741	0.116
	To male	0.000	0.999	0.212	0.651	0.185	0.672
Status							
	Redd holding	0.107	0.798	1.047	0.321	3.231	0.090
	Courted by male(s)	43.335	0.096	4.372	0.052	4.407	0.051
Duration							
	Onset of spawning	18.700	0.145	0.101	0.754	0.843	0.371
	Duration of spawning	83.596	0.069	2.354	0.143	0.024	0.879
Production							
	Number of nests	0.688	0.559	0.386	0.543	2.771	0.114
	Eggs deposited	0.643	0.570	8.025	0.011	2.204	0.156

Table 2. Results of the two-way ANCOVA with body size as a covariate to determine the effects of current velocity and channel side on male reproductive behavior. An asterisk indicates significance of probability (P) values at $\alpha = 0.05$.

Class	DV	Current Velocity		Channel		Body Size	
		F	P	F	P	F	P
Dominance							
	Dominant male ^a	1.575	0.428	0.159	0.695	0.372	0.550
	Satellite male ^b	1.040	0.494	1.895	0.187	1.068	0.316
Aggression							
	To female	1.174	0.475	0.261	0.616	0.003	0.960
	To male	210.472	0.044	2.526	0.130	2.096	0.166
Status							
	Courting	203.137	0.045	2.765	0.115	0.161	0.693

^a The number of times as dominant courting male

^b The number of times as subordinate satellite male

^c Proportion of time spent courting or holding on redd with female

Table 3. The mean (\pm standard deviation) values are shown for each response variable for the two levels of each fixed main effect included in the analysis of covariance (ANCOVA) on female reproductive behavior.

Dependent Variable	Treatment		Channel	
	High	Low	A-side	B-side
Nest Construction				
Nest Building	0.011 (0.011)	0.011 (0.014)	0.015 (0.012)	0.008(0.012)
Aggression				
To female	1.917 (3.175)	2.250 (1.960)	2.333 (3.393)	1.833 (1.528)
To male	5.417 (7.821)	5.250 (6.703)	6.333 (8.261)	4.333 (5.975)
Status				
Redd holding	0.067 (0.095)	0.062 (0.067)	0.081 (0.103)	0.048 (0.047)
Courted by male(s)	0.063 (0.070)	0.047 (0.044)	0.077 (0.072)	0.033 (0.028)
Spawning Duration				
Onset (days)	18.25 (19.68)	23.53 (16.28)	22.28 (21.80)	19.50 (13.72)
Duration (days)	3.94 (1.86)	4.09 (2.00)	3.39 (1.48)	4.64 (2.10)
Production				
Number of nests	6.42 (2.39)	7.92 (2.02)	7.42 (2.81)	6.92 (1.73)
Eggs deposited	0.984 (0.031)	0.991 (0.14)	0.977 (0.031)	0.998 (0.003)

Table 4. The mean (\pm standard deviation) values are shown for each response variable for the two levels of each fixed main effect included in the analysis of covariance (ANCOVA) on male reproductive behavior.

Dependent Variable	Treatment		Channel	
	High Velocity	Low Velocity	A-side	B-side
Dominance				
Dominant male ^a	2.75 (6.82)	6.83 (9.21)	5.42 (10.49)	4.17 (5.44)
Satellite male ^b	0.42 (1.00)	0.67 (1.07)	0.83 (1.27)	0.25 (0.62)
Aggression				
To female	8.75 (7.20)	10.92 (8.59)	10.75 (7.58)	8.92 (8.30)
To male	31.08 (36.50)	68.25 (80.08)	68.92 (82.74)	30.42 (29.03)
Status				
Courting	0.07 (0.10)	0.14 (0.17)	0.15 (0.18)	0.06 (0.07)

^a The number of times as dominant courting male

^b The number of times as subordinate satellite male

^c Proportion of time spent courting or holding on redd with female

Table 5. The frequency with which courting pairs of high velocity (HV), low velocity (LV) and anadromous males and females were observed during systematic spawning behavior observations in the Hamma Hamma River in 2002. Anadromous steelhead may have either been of purely natural origin or from releases of hatchery reared smolts in 2000.

	HV ♂	LV ♂	AN ♂	Totals
HV ♀	2	3	1	6
LV ♀	4	5	2	11
AN ♀	0	3	4	7
Totals	6	11	7	24

Table 6. Viability to the eyed stage of embryonic development for eggs hydraulically sampled from naturally constructed redds in the Hamma Hamma River in 2002.

Date	N	Viability to eye (%)	Viability to hatch (%)	Spawners observed
4/23/02	30	100	70.0	Captive female
4/23/02	93	93.5	81.7	Captive female
4/23/02	36	97.2	91.7	Captive female
4/23/02	32	96.9	84.4	Captive male
4/23/02	84	72.6	59.5	Captive male
5/15/02	29	89.7	75.9	Captive female
5/15/02	30	96.7	93.3	Captive female
5/15/02	34	97.1	97.1	Captive male
5/15/02	149	98.0	94.0	Captive male
5/15/02	475	99.2	98.5	Unknown
Average	99	94.1	84.6	

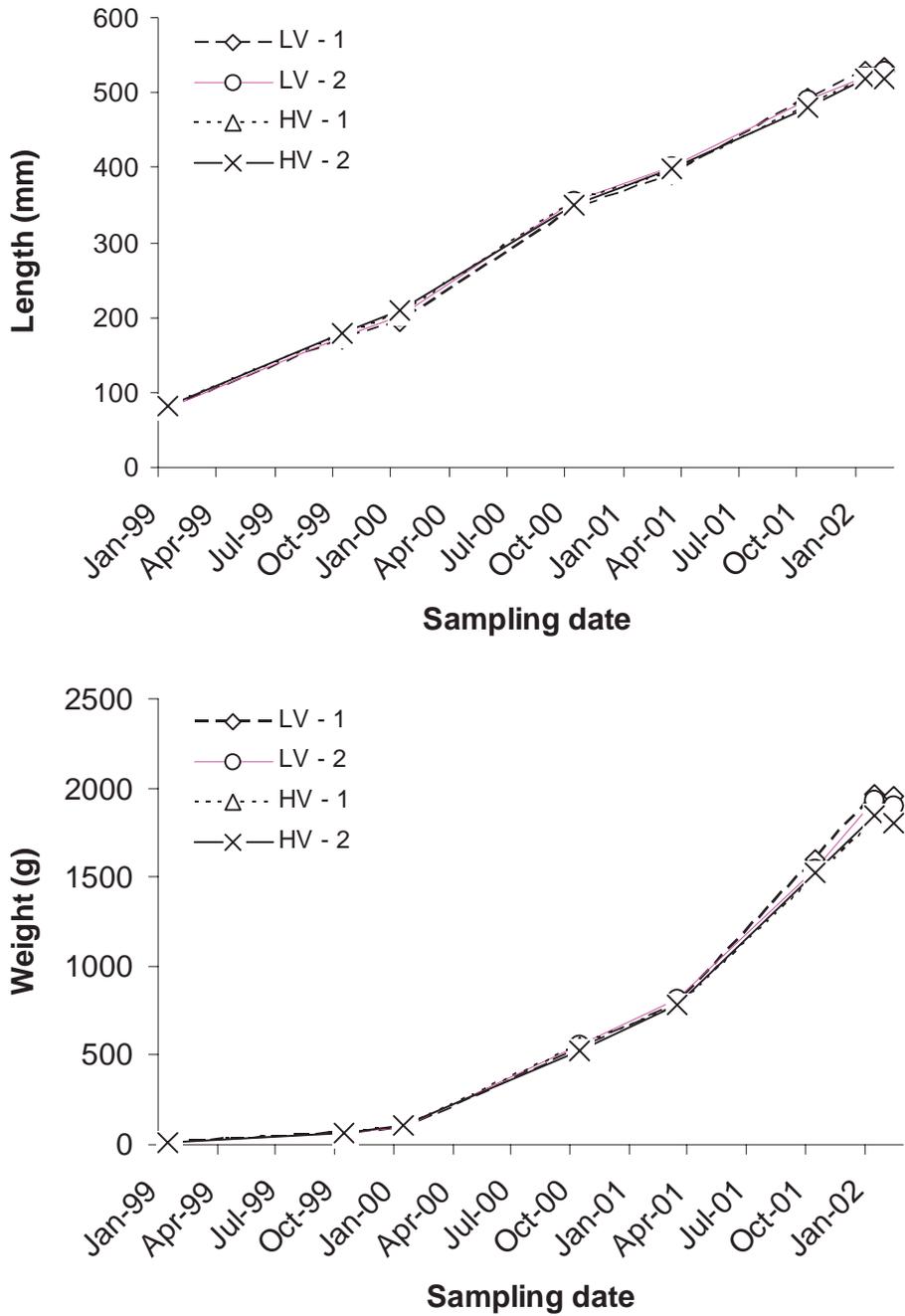


Figure 1. Length (top panel) and weight (bottom panel) of the BY 1998 captively reared steelhead from the time they were divided into the four 6-m diameter tanks in October 1999 until age-4 maturity. The four tanks are shown separately (low velocity, tank 1 = LV-1; low velocity, tank 2 = LV-2; high velocity, tank 1 = HV-1; and high velocity, tank 2 = HV-2).

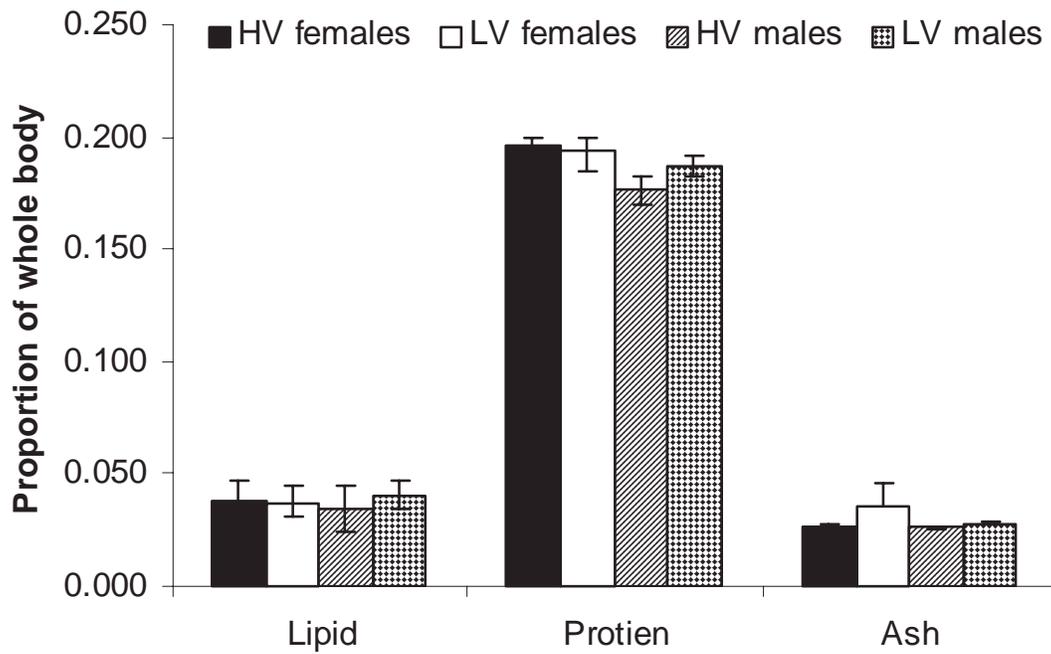


Figure 2. Proportion of the whole body composed of lipid, protein and ash (on a percent wet weight basis) for steelhead reared in high velocity (HV) and low velocity (LV) rearing vessels. Fish were collected from the spawning channel after all spawning was complete.

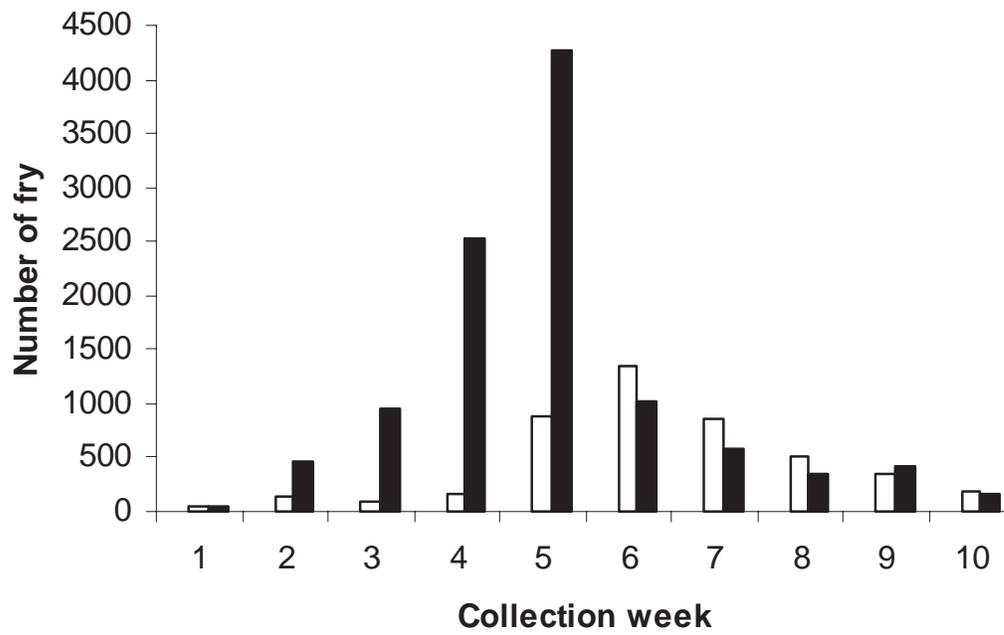


Figure 3. Number of fry collected per week in the traps located at the downstream end of the Manchester stream channel (side A = white bars; side B =black bars).

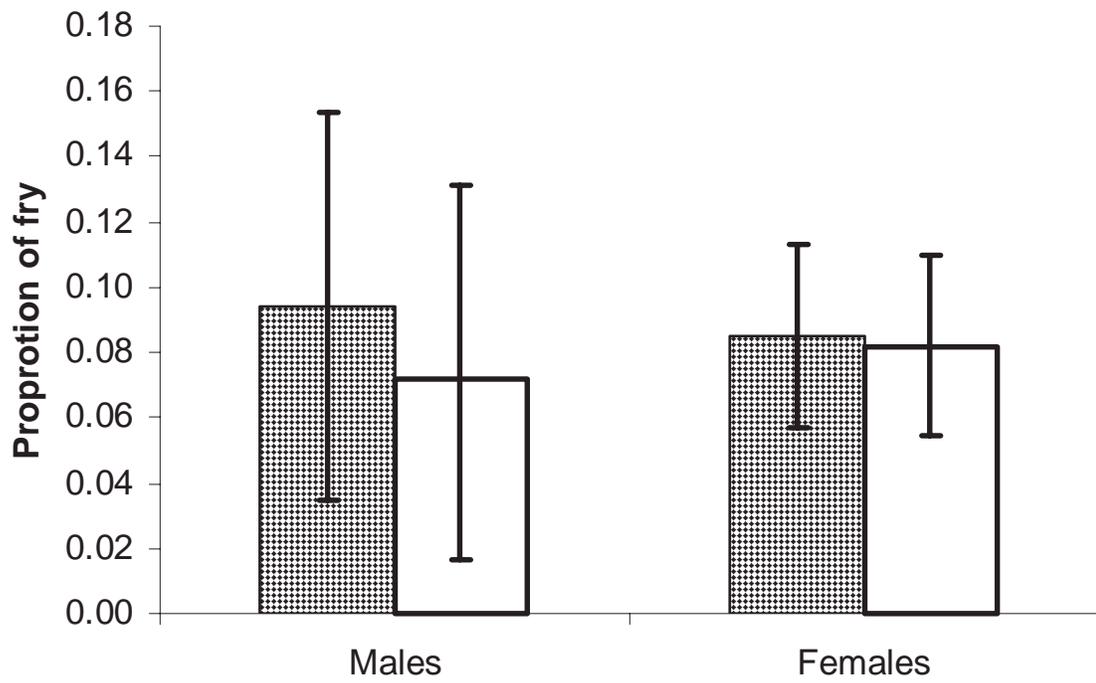


Figure 4. The mean proportion (± 2 S.E.) of fry produced from steelhead males and females reared in the high velocity (HV, clear bars) and low velocity (LV, stippled bars) rearing tanks. Proportions were calculated separately within each of the two channel sections.

References

- Barrett, B.A., and B.A. McKeown. 1988. Sustained exercise augments long-term starvation increases in plasma growth hormone in the steelhead trout, *Salmo gairdneri*. *Can. J. Zool.* 66: 853-855.
- Berejikian, B.A., E.P. Tezak, L. Park, S.L. Schroder, E.P. Beall, and E. LaHood. 2001a. Male dominance and spawning behavior of captively-reared and wild coho salmon (*Oncorhynchus kisutch*). *Can. J. Fish Aquat. Sci.* 58:804-810.
- Berejikian, B.A., E.P. Tezak, and S.L. Schroder. 2001b. Reproductive behavior and breeding success of captively-reared chinook salmon (*Oncorhynchus tshawytscha*). *N. Am. J. Fish. Manage.* 21:255-260.
- Berejikian, B.A., E.P. Tezak, S.L. Schroder, C.M. Knudsen, and J.J. Hard. 1997. Reproductive behavioral interactions between spawning wild and captively-reared coho salmon (*Oncorhynchus kisutch*). *ICES J. Mar. Sci.* 54:1040-1050.
- Berejikian, B.A. Release of captively-reared adult salmon for use in recovery. *World Aquac.* 32:1:62-64.
- Foote, C.J., G.S. Brown, and C.C. Wood. 1997. Spawning success of males using alternative mating tactics in sockeye salmon, *Oncorhynchus nerka*. *Can. J. Fish. Aquat. Sci.* 54:1785-1795.
- Houlihan, D., and P. Laurent. 1987. Effects of exercise training on the performance, growth, and protein turnover of rainbow trout (*Salmo gairdneri*). *Can. J. Fish. Aquat. Sci.* 44:1614-1621.
- Jorgensen, E.H., and M. Jobling. 1993. The effects of exercise on growth, food utilization and osmoregulatory capacity of juvenile Atlantic salmon, *Salmo salar*. *Aquaculture* 116:233-246.
- Leon, K.A. 1986. Effect of exercise on feed consumption, growth, food conversion and stamina of brook trout. *Prog. Fish-Cult.* 48:43-46.
- McDonald, D.G., C.L. Milligan, W.J. McFarlane, S. Croke, S. Currie, B. Hooke, R.B. Angus, B.L. Tufts, and K. Davidson. 1997. Condition and performance of juvenile Atlantic salmon (*Salmo salar*): effects of rearing practices on hatchery fish and comparison with wild fish. *Can. J. Fish. Aquat. Sci.* 55:1208-1219.
- NMFS (National Marine Fisheries Service). 2000. Conservation of Columbia Basin Fish: Draft Basin-wide Salmon Recovery Strategy. Prepared in consultation with the Federal Caucus. Volume 2: 179 p.

OBJECTIVE 1 - IMPROVE NATURAL REPRODUCTIVE PERFORMANCE

TASK 2. EVALUATE AND MONITOR THE BREEDING BEHAVIOR AND SUCCESS OF ESA-LISTED CAPTIVELY-REARED CHINOOK SALMON IN IDAHO STREAMS

By

Barry Berejikian and Chris Tatara

Introduction

The release of sexually mature chinook salmon reared in captivity is the primary supplementation strategy for three populations of endangered chinook salmon in Idaho. Monitoring of adult releases in 1998 - 2001 revealed that captive-reared chinook salmon mature approximately 2 - 5 weeks later than wild fish. Successful supplementation of these endangered populations depends on synchronizing the maturation of captive chinook salmon with wild chinook salmon so that the populations interbreed and their offspring experience natural emergence timing.

Materials and Methods, including Description of Study Area

The IDFG manipulated (lowered) the water temperature of captive chinook salmon in an attempt to synchronize maturation of the captive fish with the wild fish. The West Fork Yankee Fork Salmon River captive chinook population was split into two groups. One group was reared at ambient water temperatures (control) and the other was reared at temperatures below ambient conditions (treatment) at the IDFG Eagle Fish Hatchery. Rearing temperatures were maintained at these levels from the time the fish were transferred to Idaho (June 2002) until they were released for spawning into their natal stream. The spawn timing and behavior for the control and treatment groups of captive chinook salmon were monitored after release to determine the success of temperature manipulation in hastening maturation of captive fish to achieve synchronization with wild fish.

Work conducted during the FY 2002 performance period included development of a monitoring protocol to determine whether temperature manipulation of the rearing environment affected spawn timing after release through direct observation of spawning behaviors (nest digging in females and courtship in males). The final monitoring design and study methods and all data collected by NMFS researchers has been delivered to IDFG. Reporting of data analyses and results will be made by IDFG through their annual reporting process. The following summarizes work performed and some of the information collected. The FY 2002 IDFG annual report to BPA should be consulted for final study results.

Adult fish were measured, weighed, and tagged with PIT tags and color-coded, individually numbered disk tags (indicating rearing treatment) by IDFG prior to release in the wild. On August 8, 2002, 215 captive spring chinook salmon were released into a 6-mile reach of the West Fork Yankee Fork of the Salmon River located in the Challis National Forest in Idaho. The 215 fish consisted of 75 precocial males (jacks), 78 fully mature males, and 62 females. The behavioral observation protocol had two parts, and all observations were made during daylight hours. Each morning, project biologists walked and scanned the 6-mile reach of river where the captive chinook salmon were released and recorded the location and reproductive status of each fish. The scan data was then used to identify fish that were likely to spawn so that detailed (focal) observations of spawning behavior could be made from the stream bank for these fish.

Results and Discussion

The salmon reared in captivity spawned several weeks later than the wild salmon, regardless of their rearing temperature treatment prior to release. Of the 62 females released, 26 spawned to produce 33 redds, indicating that some females produced more than one redd. One NMFS biologist assisted IDFG with observations of spawning behavior in late August and early September. During that period, NMFS personnel observed spawning and construction of 3 of the 33 redds produced by the released captive fish. NMFS personnel aided IDFG in the development of the behavioral observation protocols and were responsible for collecting 9% of the spawning behavior data used for evaluating the success of releasing adults reared in captivity to supplement endangered salmon populations.

Data Management Activities

Data are collected by NOAA and PSMFC researchers onto preformatted data sheets or directly into electronic spreadsheets or text files. Data are entered and summarized on personal computers operated by researchers; primary software used for these procedures includes Microsoft Excel 2000 and Word 2000. Systat 10 is used for statistical analyses. All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report under Materials and Methods. Data analyses are reported in the Results section of the annual report. Data analyses that are incomplete will be included in the subsequent annual report(s).

Summary and Conclusions

NMFS involvement in this and previous spawning investigations in collaboration with IDFG has made clear the need to develop new technologies (tagging and recording devices) to quantify spawning behavior and breeding success. As a result, research on the calibration of electromyogram tags has been included in the FY 2003 Statement of Work for Project 199305600 (Objective 1). Further development will increase the power of field studies designed to determine the efficacy of the adult release strategy for reintroducing captively-reared chinook salmon.

OBJECTIVE 2 - IMPROVE OLFACTORY IMPRINTING

TASK 3. DETERMINE CRITICAL IMPRINTING PERIODS FOR SOCKEYE SALMON

By

Andrew H. Dittman

Introduction

Pacific salmon are well known for their ability to learn (or imprint) to odors associated with their natal stream as juveniles and then later use these retained odor memories to guide the final phases of their homestream migration. The imprinting process is critical for successful completion of the spawning migration and salmon that do not experience their natal water during appropriate juvenile stages are more likely to stray to non-natal sites. Straying by captively-reared salmon may jeopardize efforts to enhance endangered populations by either lowering the effective number of spawning adults in a captively-reared target population or via competition and interbreeding of hatchery-reared salmon with endangered wild populations.

Juvenile salmon learn odors associated with their home stream before migrating to sea, and these odors guide adult salmon during their return migrations to their natal streams. Experimental evidence has indicated that olfactory imprinting in coho salmon occurs during a sensitive period associated with a surge in plasma thyroxine levels during the parr-smolt transformation (Dittman et al. 1996). However, the freshwater rearing patterns of sockeye salmon are much more complex and the critical periods for imprinting are not known.

In sockeye salmon, the parr-smolt transformation may occur downstream or in a different location from incubation and early rearing habitats (Groot and Margolis 1991), yet salmon migrate past areas where they had undergone the parr-smolt transformation and return within very close proximity to their natal habitat. Captive rearing programs often occur at locations that do not provide the same water source experienced by a fish in its natural habitat, and therefore the timing of release may influence homing ability. Juvenile release strategies for captively-reared sockeye salmon must not only maximize survival but also minimize subsequent adult straying by ensuring that juveniles have successfully imprinted. The timing of imprinting and the effects of artificial incubation and early rearing environments on imprinting must be determined before release strategies that minimize straying can be developed.

One example illustrating some of the challenges for a captive broodstock - conservation hatchery program is the Redfish Lake sockeye salmon captive broodstock program.

Snake River sockeye salmon were listed as endangered by the National Marine Fisheries Service (NMFS) in 1991, and in that same year the Idaho Department of Fish and Game (IDFG) initiated a captive broodstock program with the ultimate goal of re-establishing sustainable sockeye runs to Stanley Basin waters. Captively-reared population numbers have increased to the point where, since 1993, fish have been re-introduced annually into the Stanley Basin. To avoid unanticipated negative consequences of any one reintroduction approach, the IDFG, in conjunction with the Stanley Basin Sockeye Technical Oversight Committee (SBSTOC), has adopted a 'spread-the-risk' strategy for reintroducing sockeye back into the wild which includes, (i) planting of eyed eggs, net pen, and direct lake releases of pre-smolts, (ii) smolt releases, and (iii) releasing captively-reared adults to spawn naturally. Fish for these releases were reared at several out-of-basin hatcheries, such as the Manchester Research Station and Big Beef Creek Hatchery in Washington, Eagle and Sawtooth Hatcheries in Idaho, the Bonneville Hatchery in Oregon, as there were no appropriate facilities within the Stanley Basin and to avoid the risk of cataclysmic events at a single facility. In some instances fish were transferred several times at different life stages between facilities, and some groups did not experience Stanley Basin waters until they were released as smolts.

This strategy is necessitated in part by the lack of knowledge about the physiological and developmental processes underlying olfactory imprinting and the ecological factors that facilitate successful homing. For sockeye salmon, reintroductions to be successful in the Stanley Basin (and throughout the Columbia Basin), fish must be released at appropriate juvenile stages for successful imprinting. Empirical studies have provided some general rules regarding the effect of hatchery rearing and release strategies on straying (Quinn 1993), but in many cases differences between species, watersheds, physical environment of the hatchery, release timing and location, and even basic assumptions about what should be regarded as successful homing may mask the underlying processes critical for imprinting and homing. Determining the critical development periods and environmental conditions for imprinting for the different salmon species will be crucial for the development and implementation of rearing and release strategies to maximize survival without increasing straying.

Materials, Methods, and Description of Study Area

Experiments to determine the critical period(s) for olfactory imprinting by sockeye salmon were initiated in fall 2000. After emergence from their natal gravel, sockeye salmon migrate to or remain in a lake where they reside for 1 or 2 years before smolting and migrating to sea. To determine the relative importance of odorant exposure during these key developmental periods, sockeye salmon are being exposed to specific odorants as alevins/emergent fry (the period just prior to and during emergence from the natal gravel (February, 2001), or as smolts (March-May, 2002).

Assessment of imprinting will be conducted in fall 2003 by measuring olfactory sensitivity to exposure odorants using behavioral assays and electro-olfactograms (EOG), a relatively simple electrophysiological technique used extensively in fishes to measure

the sensitivity of the olfactory epithelium to specific odorants (Hara 1992, Sorensen and Caprio 1997). EOG responses reflect the summated responses of many receptor neurons in the olfactory epithelium (Ottoson 1971). Because the olfactory epithelium is apparently sensitized to specific odors during imprinting (Nevitt et al. 1994, Dittman et al. 1997), the EOG may provide a rapid, sensitive, and cost-effective method for assessing sensitization to imprinted odorants.

Results and Discussion

A total of 4000 Columbia River sockeye salmon were obtained from the Colville Tribe Cassimer Bar Salmon Hatchery (eyed eggs) in November 2000. This population was chosen as a surrogate for endangered Redfish Lake sockeye in part because they have a relative extensive migration downstream, and upstream migration through the Columbia Basin and hydroelectric facilities.

Embryos were transferred to the NMFS Northwest Fisheries Science Center and reared in chilled dechlorinated Seattle City water. Subsequently, they were divided into three treatment groups, (i) alevins/emergent fry exposure, (ii) smolt exposure, and (iii) control. The smolt exposure group was further divided into 3 groups with different exposure lengths. The alevins/emergent fry were continually exposed to a mixture of imprinting odorants (phenylethyl alcohol (PEA), L - arginine, L - threonine, and L – glutamate) at a final concentration of 100 nM each from February 1 to March 5, 2001. PEA has been used extensively as an odorant for studying imprinting (Hasler and Scholz 1983; Nevitt et al. 1994; Dittman et al. 1996, 1997). Amino acid odorants have also been used in imprinting studies (Morin et al 1989) and the three amino acids used in this study represent potent odorants that activate distinct receptor types in the olfactory epithelium (Hara 1992). The use of these odorants anticipates the future development of new molecular assays for olfactory imprinting (see below).

Fish were moved to Big Beef Creek in August 2001, and will be maintained there until the end of the experiment. Beginning in February 2002 until June 1, 2002, 12 fish/treatment from each exposure group were sacrificed every three weeks for physiological sampling of gill Na^+/K^+ ATPase activity (McCormick 1993) and plasma thyroxine (Dickhoff et al. 1982) to assess smolting. The experimental fish demonstrated a >two-fold increase in gill Na^+/K^+ ATPase activity, suggesting that these fish successfully smolted during the imprinting period (Figure 1). To assess how long fish need to experience their natal water prior to release, the smolt exposure groups were continuously exposed to the imprinting odorants for six weeks (April 15 – May 24, 2002); one week (April 29 – May 24, 2002); or 1 day (May 24, 2002). These exposures are designed to approximate natural releases of fish into a lake the spring prior to smolting (6 weeks), releases of smolts into a lake (1 week); releases of smolts into outlet streams (1 day). All groups were maintained separately until after the parr-smolt transformation (May 31, 2002), and then marked by treatment. They are now being reared communally to maturity.

Assessment of imprinting in sockeye will begin in Fall 2003 by measuring olfactory sensitivity to exposure odorants using electro-olfactograms (EOG), a relatively simple electrophysiological technique used extensively in fishes to measure the sensitivity of the olfactory epithelium to specific odorants (Hara 1992, Sorensen and Caprio 1997). EOG responses reflect the summated responses of many receptor neurons in the olfactory epithelium (Ottoson 1971). Because the olfactory epithelium is apparently sensitized to specific odors during imprinting (Nevitt et al. 1994, Dittman et al. 1997), the EOG may provide a rapid, sensitive, and cost-effective method for assessing sensitization to imprinted odorants. In brief, the EOG technique involves placing a recording electrode near the medial surface of one lamellae within the olfactory rosette and monitoring electrical activity in response to perfusion of the rosette with odorants at different concentrations.

Development and testing to ready the EOG apparatus for testing odor-exposed sockeye was initiated during 2001. Initial results using coho salmon, a species for which sensitive periods for imprinting have been identified, indicated that the EOG technique is effective for measuring olfactory sensitivity to exposure odorants. Perfusion of the olfactory epithelium with the amino acids (e.g. L-arginine (Figure 2), bile acids (e.g. Taurocholic acid (Figure 3), and artificial odorants elicits concentration-dependent EOG responses.

By Fall 2002, the coho salmon exposed to amino acid odorants during sensitive imprinting periods demonstrated a heightened sensitivity to these odorants relative to control fish. Smolting coho salmon were exposed to (i) dechlorinated Seattle city water as control; (ii) control water + 10^{-7} M L-arginine; and (iii) control water + 10^{-8} M taurocholic acid (TCA) from April 1 to May 30, 200. Fish were not exposed to these odorants again until EOG testing 18 months later. All fish were PIT tagged to identify treatment and reared communally until tests for EOG sensitization to imprinted odors were conducted using maturing fish in December 2002.

Odor sensitivity was determined by measuring the initial peak amplitude of EOG responses to each odorant concentration. For comparisons of odor sensitivity, peak responses were expressed as a percentage of the response to a standard odorant (10^{-5} M L-serine), and then averaged. The standard was tested before and after each odorant dose-response profile. Fish that were exposed to the odorant L-arginine during smolting demonstrated a heightened sensitivity of the olfactory epithelium to this odorant relative to TCA-exposed fish and fish that experienced only control water during smolting (ANOVA $p = .0064$) (Figure 4).

Heightened olfactory sensitivity in the arginine-exposed fish was specific for arginine. As EOG responses to another odorant, TCA did not differ from control fish (Figure 5). TCA-exposed fish did not demonstrate heightened EOG sensitivity to TCA. This is consistent with the recent findings that amino acids but not bile acids may act as homing cues for salmon (Shoji et al. 2000).

Olfactory rosettes from all sacrificed fish were collected and frozen for later analysis of odorant receptor mRNA levels. Under another project, a quantitative PCR assay is being

developed for measuring receptor mRNA, the coho salmon orthologue of the goldfish arginine odorant receptor. This molecular approach for assessing olfactory sensitivity may provide a molecular assay for olfactory imprinting and, if successful, might be adapted easily to this study.

There is evidence that salmon must undergo sexual maturity to demonstrate heightened olfactory sensitivity and behavioral attraction to imprinted odors (Hasler and Scholz 1983, Dittman et al. 1997). Therefore, the majority of the behavioral and EOG evaluations of olfactory imprinting will be conducted in fall 2003 when these fish are expected to mature. While a few early maturing males (W. McAuley, NMFS, personal communication) may be available in September 2002 to test for the importance of maturation for recognition of imprinted odorants, the requirement for significant numbers of maturing fish (and therefore full-life cycle rearing) to test for imprinting is a major obstacle for development of routine assays for imprinting.

Recent studies suggest that the maturational hormone GnRH can stimulate migratory behavior in homing salmon (Dittman, unpublished), and heighten olfactory sensitivity (Eisthen et al. 2000). Using coho salmon exposed to imprinted odorants during smolting (March - May 2001), the known critical period for imprinting in hatchery-reared coho, the efficacy of GnRH analog implants was tested for inducing EOG olfactory sensitivity to imprinting odorants in odorant-exposed fish. EOG implants had no apparent effect on sensitivity to imprinted odorants but direct application of this hormone to the olfactory epithelium resulted in increased sensitivity to a number of odorants. Complete analysis of these results and follow-up studies to confirm this phenomenon and to eliminate potential instrumental artifacts will be reported in the next Annual Report.

Behavioral testing and EOG testing of odorant-exposed sockeyesalmon will begin again in Fall 2003. Odorant recognition and attraction experiments will be conducted in a two-choice maze similar to that described in Dittman (1994). Briefly, maturing salmon will be released into a downstream section of the maze and traps in each arm of the maze will allow fish to move upstream into either arm but not allow them to leave. Exposure odors will be continuously pumped into one arm of the maze to a final concentration equivalent to the concentrations fish experienced as juveniles. Each day fish making choices will be removed, identified, and arm choice will be recorded. Assuming behavioral responses to imprinted odors will be similar those previously observed for coho salmon (Nevitt et al. 1994, Dittman et al 1996), power analysis ($\alpha = .05$) indicates that approximately 75 fish from each experimental group will be needed for behavioral testing. Data analysis and final reports for this Task will be completed in 2004.

In Fall 2002, Stanley Basin sockeye salmon eggs were obtained to initiate a second experimental group of odorant-exposed fish for studying the timing of imprinting and the effects of artificial incubation and early rearing environments on imprinting. Particular emphasis was placed on experimental treatments that parallel rearing and release strategies that are being tested as part of the Snake River Sockeye Salmon Captive Broodstock program (BPA Project # 199107200).

Based on the current and projected release strategies for Stanley Basin sockeye the following odor exposure experiments have been initiated: Stanley Basin sockeye embryos were transferred to the Big Beef Creek and reared in constant 10°C well water. Fish will be exposed to imprinting odorants for longer durations to parallel release strategies utilized in Stanley Basin. Specifically, fish will be divided into four treatment groups, (i) eyed egg to smolt exposure (odor exposures history similar to eyed egg plants and naturally produced fish); (ii) fry to smolt exposure (similar to Sawtooth hatchery rearing and smolt release); (iii) pre-smolt to smolt exposure (similar to fall presmolt releases into Stanley Basin lakes); and (iv) brief smolt exposure (Bonneville/Eagle hatchery rearing and smolt release).

For the long-term odor exposure experiments, a single amino acid odor (L-arginine) is being used because the expense of amino acid odorants precludes long-term exposures to complex mixtures of these defined odorants. The eyed egg to smolt and fry to smolt exposure groups have been established by continual metering of L-arginine into rearing tanks to maintain a 10^{-7} M concentration. After odor exposure, fish will be marked and reared communally at Big Beef Creek. EOG and molecular testing for imprinting will be conducted throughout 2003-2005. Behavioral testing of maturing fish will be conducted in fall 2005.

Data Management Activities

Data have been collected by research staff from NMFS, the University of Washington, and the Pacific States Fisheries management Council (PSFMC) onto preformatted data sheets and entered directly into their PCs. All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report.

Summary and Conclusions

Reintroduction of captively-reared fish into the wild at inappropriate developmental periods or insufficient periods of exposure to appropriate olfactory cues may result in elevated levels of straying. The overall goal of this research is to identify hatchery practices which influence olfactory imprinting, thereby develop strategies to minimize straying of artificially produced salmonids. By identifying developmental periods important for olfactory imprinting, rearing and release strategies for each salmon species can be developed to lower stray rates in both production and recovery hatcheries. To determine the critical period(s) for imprinting for sockeye salmon, juvenile salmon are being exposed to known odorants at key developmental stages. Subsequently they will be tested for development of long-term memories of these odorants. In 2002-2003, the efficacy of EOG analysis for assessing imprinting was demonstrated, and will be applied in these and other behavioral and molecular tools in the current work plan. Results of these experiments will be important to determine the critical periods for imprinting for the offspring of captively-reared fish destined for release into natal rivers or lakes.

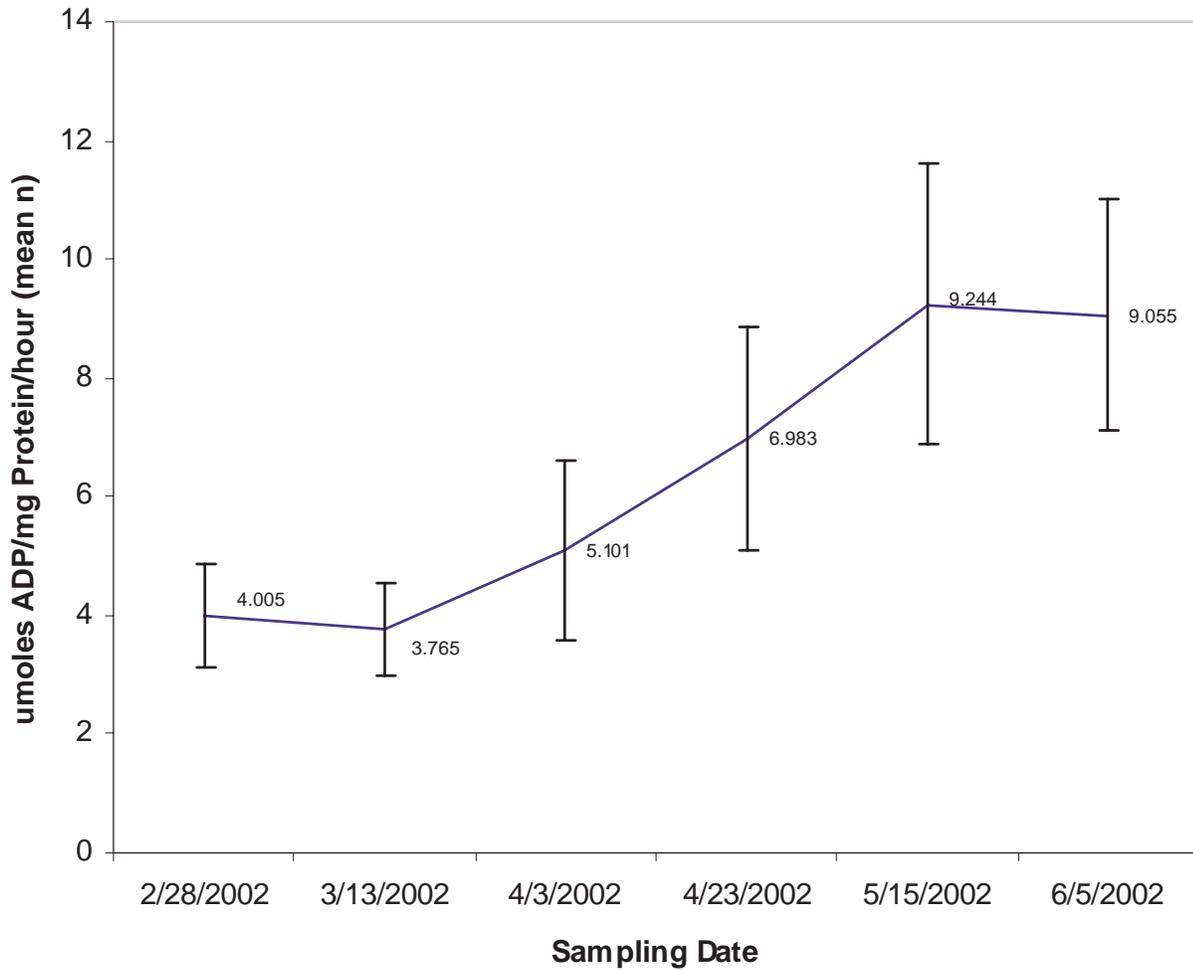


Figure 1. Assessment of smolting in Okanogan River Sockeye salmon as evidenced by Na⁺/K⁺ ATPase activity during Spring 2002.

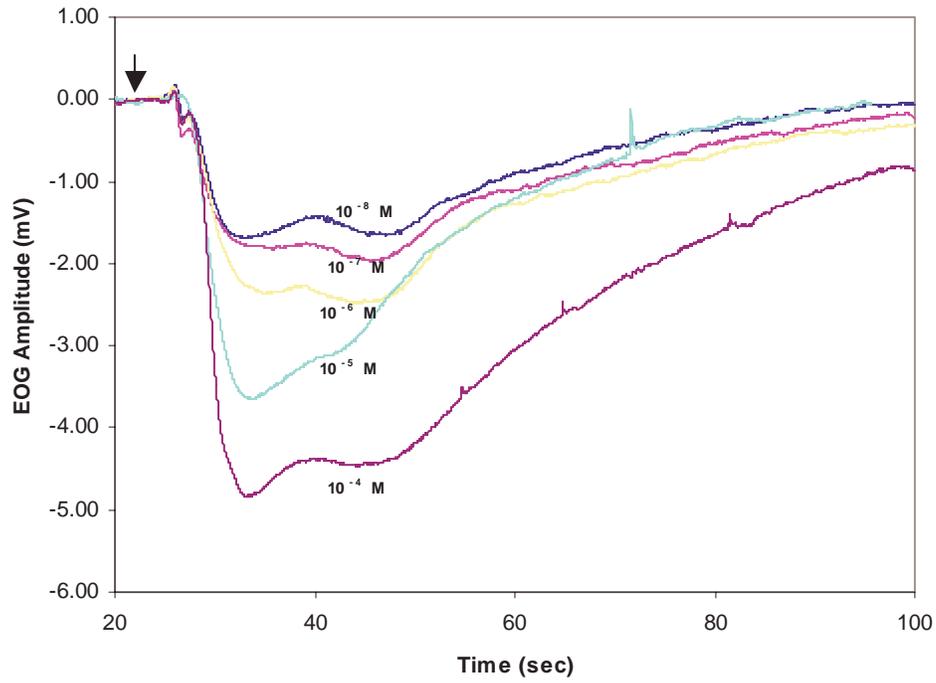


Figure 2. Representative L-Arginine EOG dose-response from 2 year-old coho salmon.

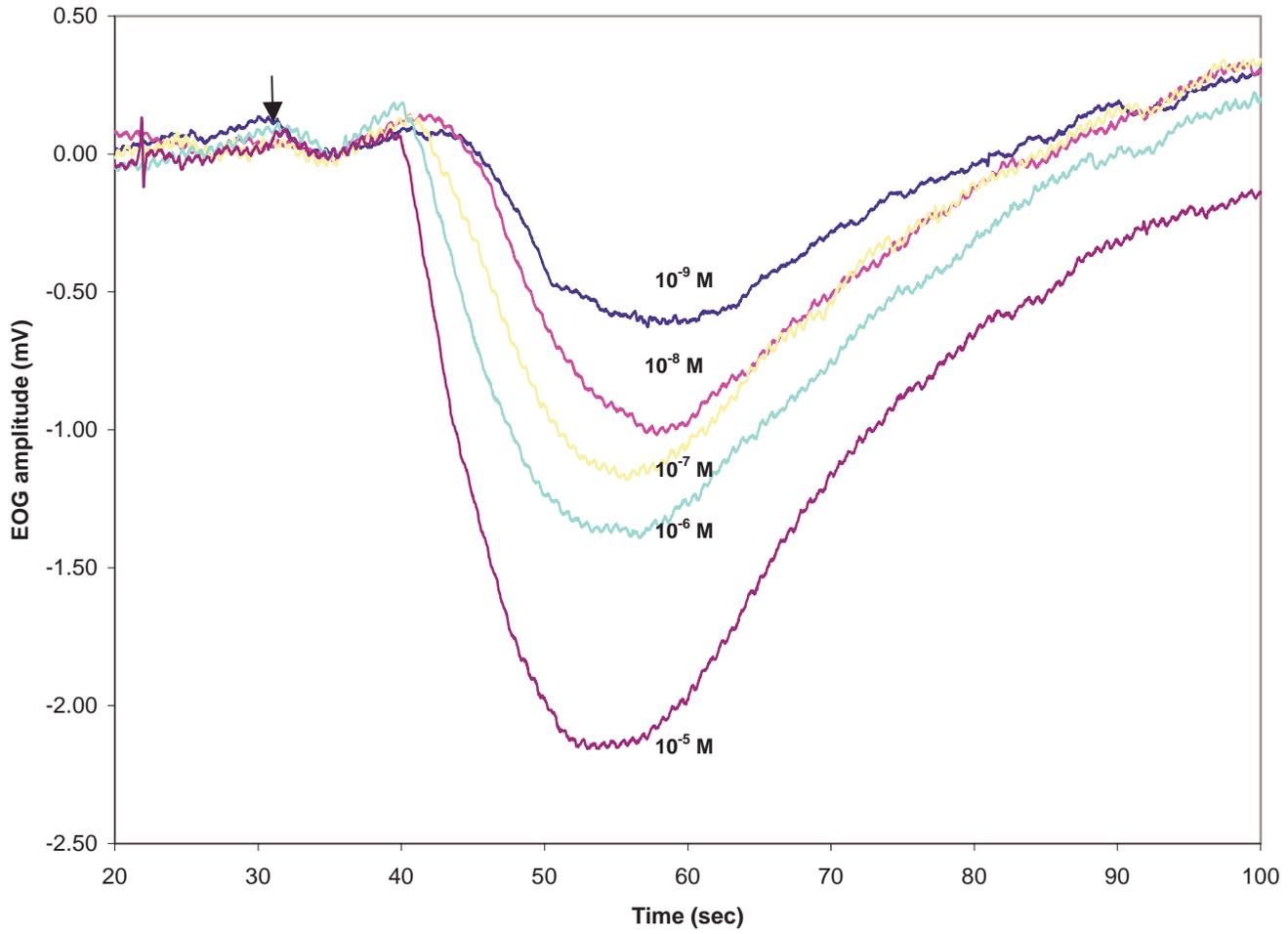


Figure 3. Representative Taurocholic Acid (TCA) EOG dose-response from 2 year-old coho salmon.

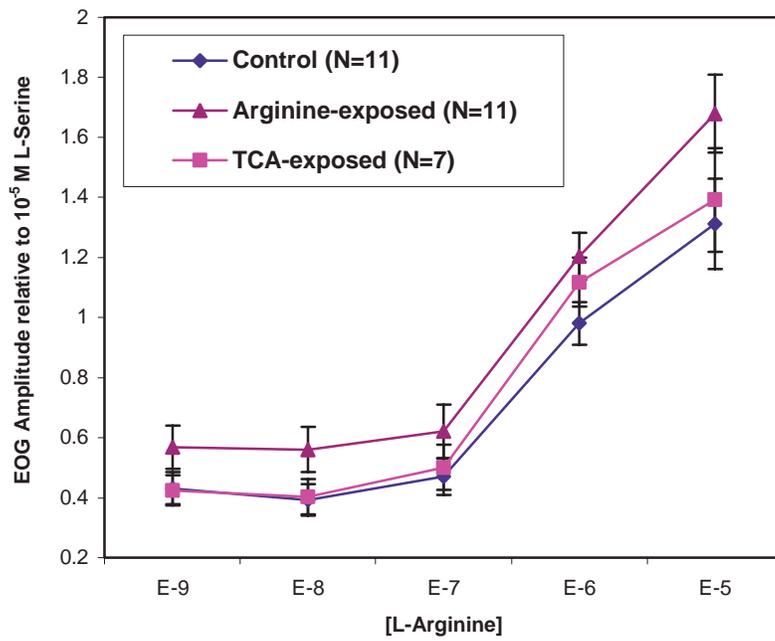


Figure 4. EOG sensitivity of Control, TCA-exposed and Arginine-exposed coho salmon to increasing concentrations of L-Arginine. Responses are expressed relative to peak responses elicited by a standard odorant (10^{-5} M L-Serine).

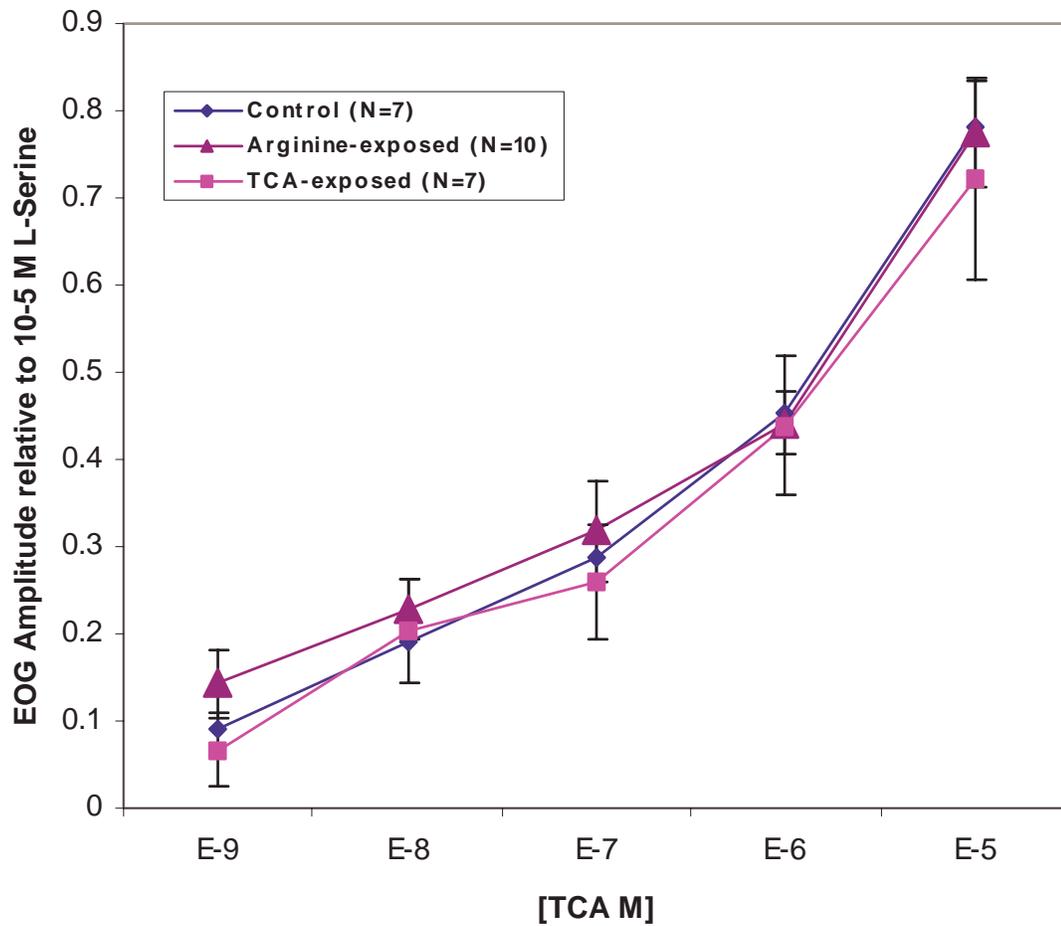


Figure 5. EOG sensitivity of Control, TCA-exposed and Arginine-exposed coho salmon to increasing concentrations of TCA. Responses are expressed relative to peak responses elicited by a standard odorant (10^{-5} M L-Serine).

References

- Dickhoff, W.W., L.C. Folmar, J.L. Mighell, and C.V.W. Mahnken. 1982. Plasma thyroid hormones during smoltification of yearling and under-yearling coho salmon and yearling chinook salmon and steelhead trout. *Aquaculture* 28:39-48.
- Dittman, A.H., T.P. Quinn, and G.A. Nevitt. 1996. Timing of imprinting to natural and artificial odors by coho salmon (*Oncorhynchus kisutch*). *Can. J. Fish. Aquat. Sci.* 53:434-442
- Dittman, A.H., T.P. Quinn, G.A. Nevitt, B. Hacker, and D.R. Storm. 1997. Sensitization of olfactory guanylyl cyclase to a specific imprinted odorant in coho salmon. *Neuron* 19:381-389.
- Eisthen, H.L., R.J. Delay, C.R. Wirsig-Wiechmann, and V.E. Dionne. 2000. Neuromodulatory effects of gonadotropin releasing hormone on olfactory receptor neurons. *J Neurosci.* 20(11):3947-55.
- Groot, C, and L. Margolis (Editors). 1991. Pacific salmon life histories. UBC Press, Vancouver, British Columbia.
- Hara, T.J. 1992. Fish Chemoreception. Chapman and Hall, London.
- Hasler, A.D., and A.T. Scholz. 1983. Olfactory imprinting and homing in salmon. Springer-Verlag, Berlin.
- McCormick, S.D. 1993. Methods for nonlethal Gill Biopsy and measurement of Na⁺, K⁺-ATPase activity. *Can. J. Fish. Aquat. Sci.* 50:656-658.
- Morin P.-P., J.J. Dodson, and F.Y. Dore. 1989. Cardiac responses to a natural odorant as evidence of a sensitive period for olfactory imprinting in young Atlantic salmon, *Salmo salar*. *Can. J. Fish. Aquat. Sci.* 46:122-130.
- Nevitt, G.A., A.H. Dittman, T.P. Quinn, and W.J. Moody. 1994. Evidence for a peripheral olfactory memory in imprinted salmon, *Oncorhynchus kisutch*. *Proc. Natl. Acad. Sci. USA* 91:4288-4292.
- Ottoson, D. 1971. The Electro-olfactogram. *In* L. M. Beidler (editor), *Handbook of Sensory Physiology*, Volume 4, Springer-Verlag, Berlin.
- Quinn, T.P. 1993. A review of homing and straying of wild and hatchery-produced salmon. *Fish. Res.* 18: 29-44.
- Shoji, T., H. Ueda, T. Ohgami, T. Sakamoto, Y. Katsuragi, K. Yamauchi, and K. Kurihara. 2000. Amino acids dissolved in stream water as possible home stream odorants for Masu salmon. *Chem. Senses* 25(5):533-540.
- Sorensen, P.W., and J. Caprio. 1997. Chemoreception in fish. *In* R.E. Evans (editor), *Fish Physiology*, CRC Press, Florida.

OBJECTIVE 3 - IMPROVE PHYSIOLOGICAL DEVELOPMENT AND MATURATION

TASK 4. THE EFFECTS OF GROWTH RATE ON INCIDENCE OF EARLY MALE MATURITY AND ADULT QUALITY IN SPRING CHINOOK SALMON

By

Penny Swanson, Paul Parkins, Brad Gadberry, Briony Campbell, Jon T. Dickey, and Karl D. Shearer

Introduction

In many salmonids, males may mature early relative to females, with the incidence varying among species, stocks, and rearing conditions for cultured fish. Although early maturing males (precocious parr, jack, or mini-jacks) are a natural phenotype for Pacific salmon such as chinook salmon, it is undesirable to produce abnormal proportions of these maturing males when fish are either released for stock enhancement or spawned in captivity in captive broodstock programs. For example, current release strategies for recovery of Snake River spring chinook salmon in particular include release of mature adult fish. Females have typically matured at age 4, while a large proportion of males are maturing at age 2 or 3. Therefore, the sex ratios of released fish have often been skewed toward females because of loss of males due to early age of maturity. In captive broodstock programs that spawn fish artificially in captivity, the asynchronous age of maturity would not be a critical problem if the quality of cryopreserved milt was reliably high, and rates of mortality were low. However, high mortality occurs in maturing 1+ age males when transferred to seawater. Because these maturing males are difficult to identify prior to seawater transfer of smolts, these individuals are frequently lost from the population. Consequently, the selective mortality of precocious males and poor quality of cryopreserved milt results in the loss of their genetic component in the population, and could reduce the effective breeding population size (N_e) of captive broodstock.

Currently, this problem is being encountered in several Snake River chinook salmon stocks presently in captive broodstock programs. The chinook salmon has a high degree of plasticity in its life cycle compared with other Pacific salmon species. Early, or precocious, male maturation can occur at several stages of the life cycle. In chinook salmon the incidence of early male maturity in cultured fish is often higher than in wild fish, and can be as high as 80% (Foote et al. 1997, Unwin and Glova 1997, Shearer et al. 2001). Early maturation of males as high as 60% has been observed in some Snake River spring chinook salmon stocks in captive broodstock programs. More recently, the incidence of early male maturity has increased as the average size of smolts has increased in the Redfish Lake sockeye salmon captive broodstock program (William Fairgrieve, NMFS, and Paul Kline, IDFG, personal communications), though it appears to be to a

lesser problem than that of chinook salmon programs. This problem is not unique to captive broodstock programs. Recently, in another BPA funded project (# 1992-02200, Physiological Assessment of Wild and Hatchery Spring Chinook Salmon) Larsen and colleagues (Larsen et al. accepted for publication) have observed high proportions of Yakima River spring chinook salmon minijacks of hatchery origin. Greater than 40% of the hatchery-produced males appear to be maturing at age 2, which is higher than observed for the wild population. Thus, there is a critical need to develop methods to control age of maturity and minimize asynchronous maturation of males and females in captive broodstock programs for threatened spring chinook and sockeye salmon, as well as in hatcheries.

The underlying factors regulating the age of maturity in salmonids involve both genetic and environmental factors. This has been investigated most extensively in rainbow trout, Atlantic salmon, and chinook salmon (Rowe and Thorpe 1990a and 1990b, Rowe et al. 1991, Silverstein et al. 1998, Shearer and Swanson 2000). The relative importance of these factors, and how they interact are poorly understood, although the environmental component appears to be strong. Because genetic selection should be minimized in a captive broodstock program for depleted stocks, rearing strategies that minimize expression of the trait are needed. It is also important to avoid strategies that alter the seasonal timing of maturation.

It may be possible to reduce the incidence of early male maturation through alteration of abiotic conditions, such as water temperature and photoperiod, or biotic factors, such as growth rates or diet composition. The approach of this research on controlling age of maturity is to identify systematically factors that influence age of maturation and to determine seasonal periods when maturation is initiated. This information is then being used to develop diets and growth regimes that allow for better control of the age of maturity, provide sufficient stored energy for appropriate life-cycle transitions, support development of gametes in adult fish, and achieve target body size for release as adult fish. In the initial studies, body fat levels were manipulated through diet, and a significant positive correlation of the percent of males maturing at 2 years of age with body fat levels was found (Shearer and Swanson 2000). Later, in an experiment in which both size and body fat levels were manipulated, growth rate or size one year prior to maturation was the primary factor affecting maturation at 2 years of age (Silverstein et al. 1998). Body adiposity influenced the rate of maturation only in small fish.

Two studies were conducted subsequently to determine the threshold of size or growth rate that influenced the onset of male maturation. In the first study (Shearer et al. 2000), fish were fed high protein, low fat diets at graded ration levels. Fish size during the first autumn ranged from 50 - 100 g, and 65 - 90% of the males matured one year later (age 2). From this study, it was concluded that, even though size or growth rate clearly influenced the rate of male maturation at age 2, the threshold had been exceeded. In the second study (Shearer et al. 2001 and 2002), fish were reared on graded rations of a commercially available diet. The feeding regime targeted a much lower body size for the first year of rearing than the first study. For chinook salmon, the combined data suggest that a growth trajectory that produces a fish of 10 g body weight in the first year of

rearing should be used to reduce maturation substantially in the subsequent year (age 2). This target size is similar to that of juvenile wild Yakima River spring chinook salmon, which exhibit lower rates of early male maturation than hatchery-reared fish of the same stock (Don Larsen, NWFSC, personal communication).

While substantial progress has been made in developing growth regimes to reduce early male maturity in chinook salmon, several questions remain to be addressed. First, what are the critical periods when accelerated growth triggers the onset of maturation? In the last two studies (Shearer et al. 2000 and 2001), fish were reared on fairly constant growth rates throughout the two years of rearing. Thus, it is not known when growth can be accelerated without triggering maturation. Because captive rearing programs have been unable to achieve wild size adults, it is imperative to know when growth can be accelerated to increase body mass and not trigger reproductive maturation. Previous work by Silverstein et al. (1998) and Swanson and Shearer (2000) suggested that this period was almost one year prior to spawning. This needs to be verified using current information on threshold size/growth rate for triggering maturation. Second, are these data more generally applicable to other stocks and species of Pacific salmon? Willamete River spring chinook salmon have been used for most of this current research on growth and male maturation. It is possible that specific thresholds for size or growth rate may vary between stocks and species. Third, what are the effects of the growth manipulations on female maturation? If these growth manipulations are applied in captive broodstock programs or hatchery programs to reduced 1+ age maturation, it is critical to determine the potential impacts on females. These questions are being addressed in the present study.

Materials, Methods, and Description of Study Area

The hypothesis is that increased growth after the spring equinox does not induce maturation in the subsequent autumn. It is speculated that a critical period exists during the autumn/winter when attainment of a threshold growth rate (or size) will initiate maturation for the following year. To determine if there are stock differences in the effects of growth on male maturation, this hypothesis is being tested using Willamete River (WR) spring chinook salmon, together with two parallel treatment groups of Rapid River (RR) spring chinook salmon.

Maturation of age 2 males in all groups, and ovarian development in the WR treatments, are being monitored. Eight growth regimes are being tested in the WR stock and two growth regimes are being tested in the RR stock. Each growth regime consists of a combination of two growth rates: (T8) with target size of 8 g, and (T60) with target size of 60 g in December 2002. The timing of the shift from T8 to T60 is varied by treatment group to encompass the hypothetical sensitive window (September -December) for initiation of maturation for the subsequent fall. Previous studies have indicated that the period when meiosis (presence of primary spermatocytes) is evident throughout the testis of maturing fish occurs by April - May, indicating that the fish are committed to maturation in the subsequent fall. It is speculated that increased growth during or after

this spring period will not initiate maturation for the fall. The design of the growth manipulations during the first two years of rearing is represented in Table 1.

Eggs from the Oregon Department of Fish and Wildlife Willamette River Salmon Hatchery and the Idaho Fish and Game Rapid River Hatchery were obtained in October 2001 and incubated at 5°C at the Northwest Fisheries Science Center hatchery facilities in Seattle, WA. At first feeding, in February 2002, the fish of each stock were placed into two 6 ft. diameter tanks with recirculated fresh water maintained at 5°C. The water temperature was gradually increased to 12°C over the next 5 months. Using the bioenergetics model of Cho (1992), a daily ration was calculated to produce fish of 8 g on December 1, 2002. The model requires inputs for the following: rearing temperature, the thermal growth coefficient (TGC), daily energy gain as a percent of the dietary energy fed (25%), heat increment of feeding (0.6), and energy digestibility (90%). The fish in all treatments are being fed the same commercial feed (BioOregon Grower, 22.1 KJ/g energy, 49% protein, 22% lipid).

Table 1. Growth regimes for experimental groups. Each growth regime consists of two growth rates: T8 (target size of 8 g) and T60 (target size of 60 g). Changes in growth will be made in the middle of indicated months. Willamete River (WR) and Rapid River (RR) spring chinook salmon will be used for the experiment. The hypothetical “sensitive” period when high growth influences the onset of maturation occurs from September to December.

Stock/Treatment	Period of low growth rate (T8)	Period of high growth rate (T60)	Predicated rate maturation of age 2 males
WRA	Feb 2002-Jul 2002	Jul 2002- Sept 2003	High
RRA	Feb 2002-Jul 2002	Jul 2002- Sept 2003	High
WRB	Feb 2002-Sept 2002	Sept 2002- Sept 2003	High
WRC	Feb 2002-Nov 2002	Nov 2002- Sept 2003	Intermediate
WRD	Feb 2002-Jan 2003	Jan 2003- Sept 2003	Intermediate
WRE	Feb 2002-Mar 2003	Mar 2003- Sept 2003	Low
WRF	Feb 2002-May 2003	May 2003- Sept 2003	Low
WRG	Feb 2002-Jul 2003	Jul 2003- Sept 2003	Low
WRH	Feb 2002-Sept 2003	None	Low
RRH	Feb 2002- Sept 2003	None	Low

WR fish have been divided into 8 treatments (WRA-H; 2 tanks per treatment), and RR fish have been divided into 2 treatments (RRA and RRH; 2 tanks per treatment). All fish are being reared on the lowest growth rate (T8) from first feeding (February 2002) through July 2002. Two groups, WRH and RRH, are being maintained on the T8 until the end of the first phase of the experiment in September 2003. Starting in July 2002, the ration in two groups, WRA and RRA, was increased to produce a growth rate comparable to one that would produce a target size of 60 g in December 2002 (T60). At two-month intervals, the ration of additional groups, WRB-WRF, was increased to produce a T60 growth rate. The ration of the WRG group will be increased in July 2003. Ration

adjustments up to T60 have been made in 2002 and 2003 on September 21 (WRB), November 21 (WRC), January 21 (WRD), March 21 (WRE), and May 21 (WRF), respectively. Fish in WRB-WRF were pair fed to the A treatment groups at the size at which they were split of from the low growth rate (T8). Sample weights are being collected bimonthly to monitor growth and adjust ration accordingly. The number of fish allocated to treatments WRB-WRG varied from 200-260/tank to accommodate terminal sampling during rearing and ensure that 160 fish remained in the treatment until the end of the experiment in Sept 2003. During the two month intervals from June 2002 through September 21, 2003 samples of 20 WR fish per tank are being collected for proximate composition, gonad histology, and plasma levels of 11-ketotestosterone (11KT) in males only, estradiol (E2) in females only, and plasma insulin-like growth factor I (IGF I) in all fish and follicle stimulating hormone (FSH) in pooled samples from fish of the same sex and stage fish. The intermediate sample collection is necessary to assess the progress of maturation, and also obtain information on the reliability of 11 KT for predicting maturation rates in male fish.

At each sampling, 20 fish from each tank were randomly selected for weight, length and body composition determination. At the end of the experiment the remaining fish will be weighed, measured, and sexed. Testes were weighed in maturing males. For proximate analysis, fish smaller than 1 g were pooled (10 fish), fish between 1 and 100 g were chopped, dried and ground in a mortar and pestle; fish larger than 100 g were ground in a food processor and a sub sample of 100 g of wet material was dried, then reground and a sub-sample was taken for analysis. Moisture was determined by drying to constant weight at 105°C. Fat was determined using a Soxhlet device with dichloromethane as the solvent. Tank means (\pm standard errors) were used as the unit of observation unless otherwise stated. Percentage data were arcsine transformed prior to ANOVA and Fisher's PLSD test was used to conduct multiple mean comparisons. Regression was performed on untransformed data. All analyses were performed using Statview™ (Abacus Concepts, Berkeley, CA, 1992).

Results and Discussion

Total mortality has been less than 0.1%, to date. Growth of the fish has closely followed the weights predicted using the project feeding protocol. The mean weights of the fish in treatments WRA, B, C, D, E and H, RRA, and RRH are shown in (Figure 1). Although there was an attempt to grow fish in the RRA group in parallel with the WRA group, fish in the RR stock did not grow as well as the WR stock. Whole body fat levels have remained low in the low ration fish (Treatment WRH), but have increased rapidly in groups of fish once growth was accelerated (Figure 2). In March 2003, maturing males in all treatments had higher levels of body fat than non-maturing males and females, which had similar levels of body fat (Figure 3). Based on visual determination of testes in male fish samples in March, the incidence of male maturation appears to be positively related to fish size (Figure 4), and to the duration of the period of accelerated growth.

Treatments G will be initiated in July 2003 and interim sampling will at this time in the all other groups. A final sampling of all remaining fish will take place in September 2003 when maturing males should be spermiating. Once all samples are collected, analyses of hormones and gonadal histology will be performed during the FY2003 funding period. It is expected that this task will be completed by June 1, 2004.

Data Management Activities

Data are collected manually by NOAA, UW, and PSMFC researchers onto preformatted data sheets or directly into PCs. Data are entered and summarized on personal computers operated by researchers using Microsoft Excel, Statview 512+, Cricket Graph 1.3.2, and Prism. Data on histology of tissues is recorded using a digital camera and stored as either TIFF or JPG files on a Power MacIntosh G4. Backup copies of data are saved on an external Lacie hard-drive and on CD-ROMs. All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report under Materials and Methods. Data analyses are reported in the Results section of the annual report.

Summary and Conclusions

Not applicable at this time.

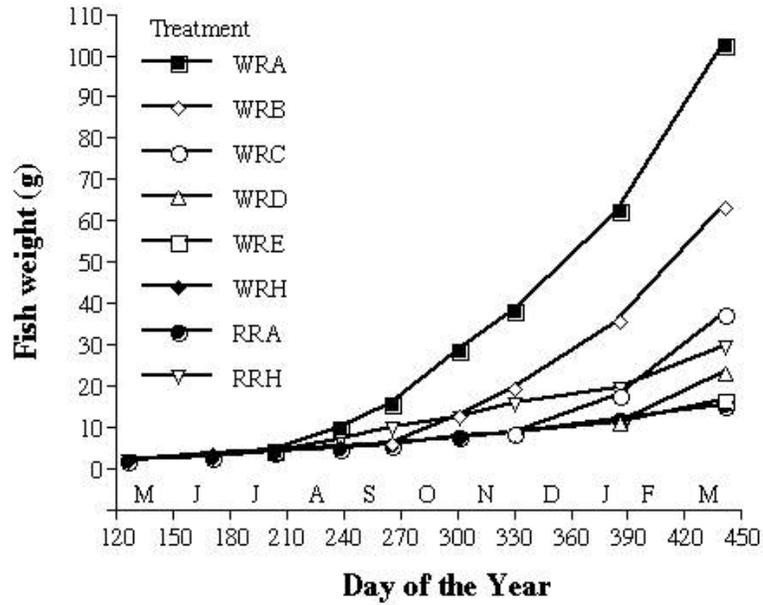


Figure 1. Body weight in all treatment groups during the experimental period. Each point is the mean of two tanks. WR, Willamete River stock; RR, Rapid River stock.

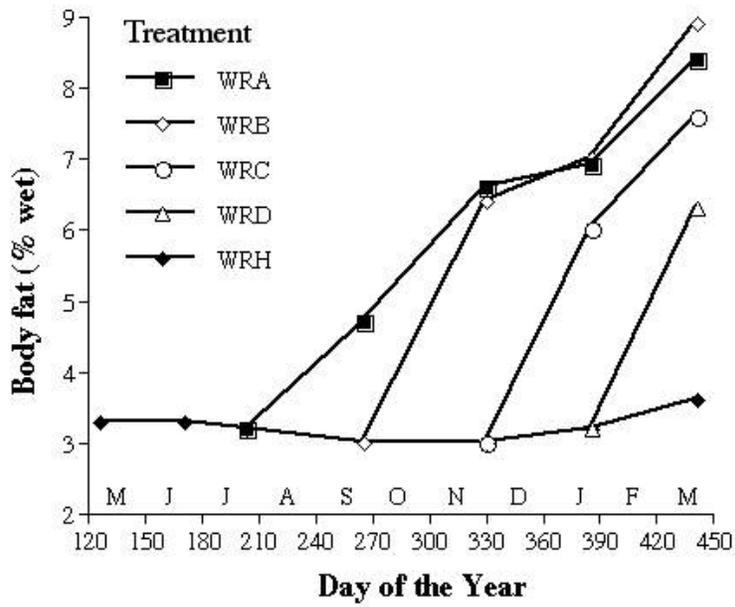


Figure 2. Whole body fat (% wet basis) in Willamete River (WR) treatments. Data are mean \pm S.E. of $n=2$ tanks. Error bars are smaller than data point symbols.

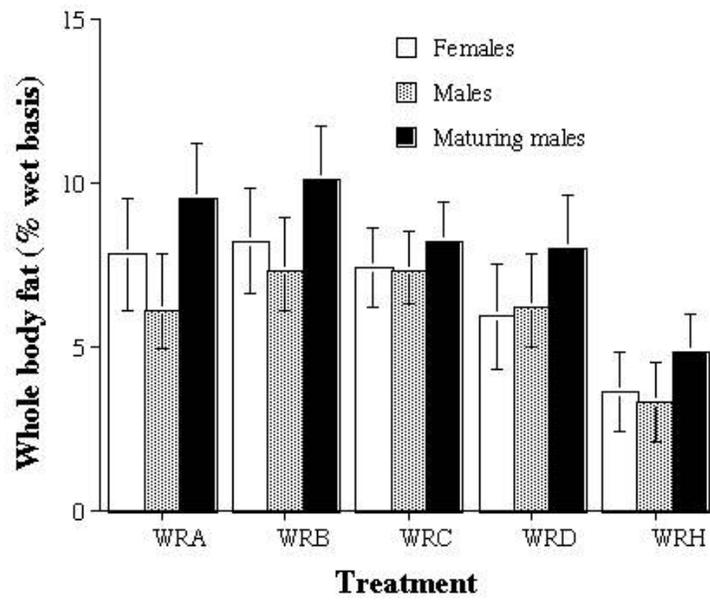


Figure 3. Whole body fat levels of Willamete River non-maturing males, maturing males and females sampled in March 2003. Data are mean +/- S.E. of n= 2 tanks.

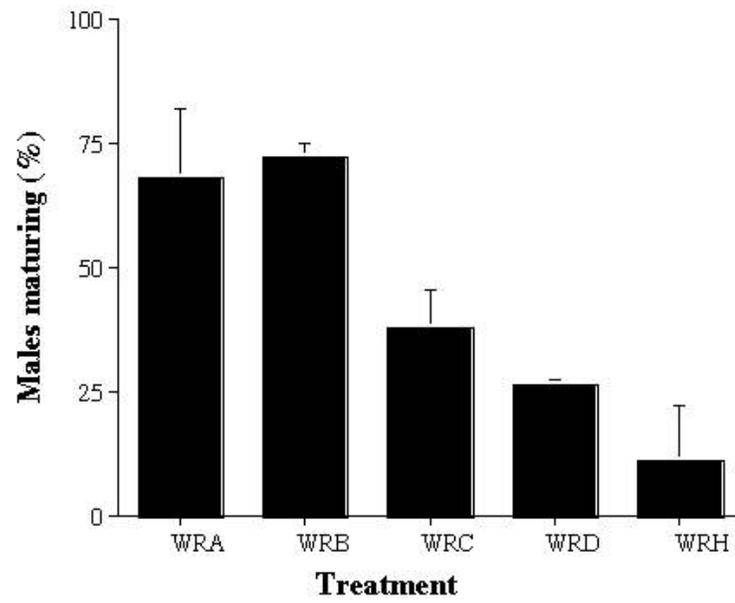


Figure 4. Percent maturing males in the Willamete River treatments sampled in March 2003. Data are mean +/- S.E. of two tanks.

References

- Cho, C.Y., 1992. Feeding systems for rainbow trout and other salmonids with reference to current estimates of energy and protein requirements. *Aquaculture* 100: 107-123.
- Foote, C.J., G.S. Brown, and C.C. Wood. 1997. Spawning success of males using alternative mating tactics in sockeye salmon, *Oncorhynchus nerka*. *Can. J. Fish. Aquat. Sci.* 54:1785-1795.
- Larsen, D.A., Beckman, B.R., Cooper, K.A., Barrett, D., Johnston, M., Swanson, P., and Dickhoff, W.W. (Accepted). Assessment of high rates of precocious male maturation in a spring chinook salmon supplementation hatchery program. *Trans. Am. Fish. Soc.*
- Rowe, D.K., and J.E. Thorpe. 1990a. Differences in growth between maturing and non-maturing male Atlantic salmon (*Salmo salar* L.) parr. *J. Fish Biol.* 36:643-658.
- Rowe, D.K., and J.E. Thorpe. 1990b. Suppression of maturation in male Atlantic salmon (*Salmo salar* L.) parr by reduction in feeding and growth during spring months. *Aquaculture* 86:291-313.
- Rowe, D.K., J.E. Thorpe, and A.M. Shanks. 1991. The role of fat stores in the maturation in male Atlantic salmon (*Salmo salar*) parr. *Can. J. Fish. Aquat. Sci.* 48:405-413.
- Shearer, K.D., and P. Swanson. 2000. The effect of whole body lipid stores on early maturation of male spring chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture* 190:343-367.
- Shearer, K.D., P. Swanson, B. Campbell, B.R. Beckman, P. Parkins, and J.T. Dickey. 2000. Effects of growth and a low fat diet on incidence of early male maturation of spring chinook salmon (*Oncorhynchus tshawytscha*). In B.A. Berejikian and C.E. Nash (editors), *Research on Captive Broodstock Programs for Pacific Salmon*, p.35-51. Annual Report to Bonneville Power Administration for FY99.
- Shearer, K.D., B. Campbell, J.T. Dickey, P. Parkins, B. Gadberry, and P. Swanson. 2001. Effects of growth and on incidence of early male maturation and adult quality of spring chinook salmon (*Oncorhynchus tshawytscha*): Progress Report. In B.A. Berejikian and C.E. Nash (editors), *Research on Captive Broodstock Programs for Pacific Salmon*. Annual Report to Bonneville Power Administration for FY00.
- Silverstein, J.T., K.D. Shearer, W.W. Dickhoff, and E.M. Plisetskaya. 1998. Effects of growth and fatness on sexual development of chinook salmon (*Oncorhynchus tshawytscha*) parr. *Can. J. Fish. Aquat. Sci.* 55:2376-2382.
- Unwin, M.J. and G.J. Glova. 1997. Changes in life history parameters in a naturally spawning population of chinook salmon (*Oncorhynchus tshawytscha*) associated with releases of hatchery-reared fish. *Can. J. Fish. Aquat. Sci.* 54:1235-1245.

OBJECTIVE 3 - IMPROVE PHYSIOLOGICAL DEVELOPMENT AND MATURATION

TASK 5. DETERMINE EFFECTS OF GROWTH ON MATURATION TIMING, FECUNDITY, EGG SIZE, AND EGG QUALITY IN COHO SALMON

By

Briony Campbell, Brian R. Beckman, William T. Fairgrieve, Jon T. Dickey, and Penny Swanson

Introduction

One of the major aims of the captive broodstock program is to optimize the production of fertile eggs from captive broodstock, which are either introduced to the wild or spawned artificially in captivity. Both the Redfish Lake sockeye and Snake River spring chinook salmon captive broodstock are reported to display dysfunctional ovarian development such as small gonads, atretic eggs, variable egg quality, reduced egg size and egg number, and delayed timing of ovulation compared with wild counterparts. Such problems result in a significant reduction in offspring production severely reducing the effectiveness of these recovery programs.

Salmonids live in a varied environment where changes in factors, such as food availability and temperature, demand flexibility in maturation strategies (life histories) to maximize reproductive success. Within a population, individuals can follow different developmental pathways, ultimately spawning at different sizes and ages (Groot and Margolis 1991). The developmental pathways available to an individual or population have been genetically selected over time to maximize reproductive success within the bounds of the environmental variability experienced during selection.

One primary life history decision is age of sexual maturity. Gonad maturation in salmonids commences approximately one year prior to spawning (Thorpe 1994, Shearer and Swanson 2000, Brooks et al. 1997). Therefore, proximate (predictive) cues are required to predict future gamete production and survival in order to select for developmental pathways that will optimize reproductive success. Current theories on the mechanisms involved in determining age of maturation propose that some aspect of growth and/or metabolic reserve is the primary predictive cue and is compared to genetically-set thresholds to determine the subsequent direction of maturation (e.g. to mature or remain immature). According to this theory, if growth exceeds this threshold at a critical time of year, maturation will proceed. Evidence suggests that, for Atlantic and chinook salmon that spawn in the fall, there are at least two periods where threshold-driven decisions are being made, one in the fall one year prior to spawning and one in the

spring 6 months prior to spawning, thus, for a fall spawning species, an ‘initiation’ period in the fall is followed by a ‘permissive’ period for continuing maturation in the spring. (see Thorpe 1998, Duston and Saunders 1997, 1999, Shearer and Swanson 2000).

In addition to affecting the direction of major developmental changes, such as maturation, evidence suggests that growth during various life stages affects allocation of body energy resources to final egg size and egg number. Analyses of data on egg size and fecundity in Pacific salmon (Healey and Heard 1984, Fleming and Gross 1990, Beacham and Murray 1993) have formed the basis of many evolutionary theories for the control of egg size (e.g. Roff 1992, Stearns 1992). Several studies have suggested that egg size in salmonids is influenced by growth rate (Fleming et al. 1996, Jonsson et al. 1996) and have associated poor growth at various developmental stages with the subsequent production of larger but fewer eggs (Thorpe et al. 1984, Jonsson et al. 1996, Lobon-Cervia et al. 1997, Morita et al. 1999, Tamate and Maekawa 2000). Recent results from the current research on maturation in female coho salmon have indicated that growth over one year prior to spawning can have significant effects on subsequent ovarian development (Campbell et al. 2002). Body size just after smoltification has been related to the decision to mature the following year (see Duston and Saunders 1997 and 1999; Campbell et al. 2002). Results from these studies indicate that decisions involving maturation, timing, and allocation of resources can be made very early in the salmon life cycle. Once such pathways are set, attempted corrective measures later in the life cycle may be ineffective.

In Pacific salmon little is known about the factors and mechanisms that control ovarian growth. In semelparous fish, the response to factors that influence the physiological commitment to ovarian growth is crucial, as there is no second opportunity once this commitment is made. Research in other fish species has shown that ovarian development involves a dynamic interaction between oocyte proliferation, growth, maturation, and death. The resulting reproductive investment is a product of these processes. The factors that cause dysfunction in ovarian development in captively-reared salmon broodstock are unknown. It is hypothesized that such abnormal development is a result of inappropriate environmental signals (e.g. food availability and temperature) during periods when females are allocating body resources towards maturation. Thus, abnormal ovarian development may be due to rearing conditions early in the life cycle when physiological commitments to egg number and size are occurring, and/or during the year prior to spawning when ovary growth commences. It is essential to understand which factors initiate ovary growth and how oocyte development is maintained through to ovulation. The ultimate goal of the broodstock programs is to optimize these processes to maximize both egg production and quality.

A large amount of information is now available on the physiological factors controlling oocyte growth during yolk deposition, which occurs relatively close to ovulation (Patino and Sullivan 2002). However, few studies have attempted to determine factors controlling the earlier stages of pre-vitellogenic growth, the period during which the decision to mature and commitments to egg size and egg number are made. A few studies have suggested a role for follicle-stimulating hormone (FSH) and sex steroids

such as 17β -estradiol in pre-vitellogenic oocyte growth (e.g. Khoo, 1979). In addition, steroidogenic acute regulatory protein (StAR) is a site of FSH control of steroid production in the ovary and is up-regulated during maturation (see Miller and Strauss 1999). These parameters are very early indicators of the initiation of ovarian maturation during pre-vitellogenic growth and to date have not been measured during the proposed critical threshold-driven decision periods in salmonids.

Materials, Methods, and Description of Study Area

Beginning in their early freshwater-rearing phase and continuing through their seawater-rearing phase, growth in coho salmon was monitored to determine critical periods when ovarian maturation is initiated and the relationship with growth during such periods. In addition, ovarian development was monitored, and several aspects of FSH and steroid production were measured, to determine if a significant up-regulation of these factors occurred during the proposed fall 'initiation' and spring 'permissive' periods for ovarian maturation. Coho salmon were selected for this study because of the simple life history and the availability of physiological tools for determining the status of maturation in this species. These data will have important implications in designing feeding and growth regimes to maximize and/or mirror natural production in Pacific salmon species.

The aims of the experiment were to (i) produce female coho salmon of a range of body sizes and growth rates, (ii) determine the point at which ovarian maturation was initiated, and (iii) determine the effect of body growth on the initiation process and subsequent ovarian development. Fertilized eggs of coho salmon (1999 brood) were obtained from the University of Washington Hatchery and reared for 14 months at the NWFSC Montlake Hatchery.

In February 2001, 1300 fish were individually tagged with PIT tags and fin tissue was collected from all fish for determination of genetic sex using a molecular marker for the Y chromosome (Du et al. 1993). This marker has been validated for use in three Puget Sound stocks of coho salmon. Using the growth model based on the delta-1 method of Piper (1982), a daily ration was calculated to produce fish of 33 g by May 2001. The model requires inputs for the following parameters: rearing temperature, the thermal growth coefficient (13.7°C temperature units), and feed-gain ratio. Feeding rates were adjusted monthly based on actual growth and feed conversion data collected during monthly samplings. Fish were fed a commercially available diet (Bio-Oregon, BioDiet Grower).

In May 2001, fish were sorted by sex and all females (620) and 100 males were moved to the NMFS Manchester Research Station. Fish were randomly split into four 12-ft diameter fiberglass tanks supplied with filtered and UV-treated seawater. A daily ration was calculated (as stated above) to produce fish of with an average body weight of 280 g by May 2002. This average size was selected in order to produce a mixture of maturing and non-maturing females, based on data obtained from a previous trial (Campbell et al. 2002). Fish were fed a commercially available diet (Moore Clark, BroodSelect).

Mortality was a major problem during this experiment, with approximately 60% of the females dying during the seawater growth phase between May 2001 and August 2001. The remaining fish were affected by an unidentified tail rot syndrome during fall 2001 and were treated with intraperitoneal injections of oxytetracycline (30mg/kg body wt.) during November 2001 and January 2002. These mortalities precluded collecting any of the proposed spawning samples that were originally planned in order to correlate final egg production with growth history. Due to the low number of surviving females the ration treatments originally proposed for the fall of 2001 could not be tested. Therefore, the experiment was altered to monitor growth and reproductive development with periodic terminal sampling up to May 2002 (see below).

Sample collection and analysis

Body weight and length were recorded monthly to monitor growth of all fish from January 2001 through January 2002, and at the final terminal sample in May 2002. To monitor ovarian development and maturation of the reproductive endocrine axis, ten to twenty females were sampled on a monthly basis from February 2001 through December 2001, and at the final terminal sample in May 2002. Fish were killed in a lethal dose of buffered tricaine methanesulfonate (MS-222, 0.05%), and body weight and length were recorded. Blood was collected via the caudal vein, initially using heparinized capillary tubes after severing the caudal peduncle, and then by venipuncture using heparinized syringes and 21 g needles once fish were > 30 g body weight. Pituitaries were frozen in liquid nitrogen and stored at -70°C . Ovaries were weighed and fixed in Bouin's fixative for 24 hrs prior to storage in 70 % ethanol. Fixed ovaries were dehydrated through ethanol and imbedded in paraplast, sectioned (4 μm), and stained with hematoxylin and eosin. Stages of oogenesis were determined by light microscopy using Bromage and Cumaranatunga (1988) as a guide. Blood was centrifuged at 1000 x g for 5 min and plasma was stored at -70°C . Plasma 17 β -estradiol was measured directly by RIA using a commercially available kit (ICN diagnostics). Pituitary FSH, and plasma FSH levels were measured by RIAs as described in Shearer and Swanson (2000). FSH-receptor and StAR transcript levels were measured by quantitative real time PCR in ovaries collected during the proposed critical fall period, and in the final sample in May 2002. Primers and probes for salmonid StAR and FSH-receptor were designed and created using the software package "Primer Express" (Applied Biosystem Inc. Foster City, CA). PCR amplification and fluorescence detection was performed using an ABI Prism 7700 Sequence Detector. Acidic Ribosomal Protein-PO was used for normalization of RNA used in reverse transcription reaction and subsequent Real-time RT-PCR TaqMan assays.

Statistics

Comparisons between parameters according to sample date were done by one-way ANOVA and PLSD was used to conduct multiple mean comparisons. Body weight, pituitary FSH, plasma 17 β -estradiol, plasma FSH were log transformed and percentage data were arcsine transformed prior to ANOVA in order to meet with test criteria. All

analyses were performed using StatviewTM (Abacus Concepts, Berkeley, CA, 1992). Data in the figures are given as mean \pm sem. Significance test level was set at $p < 0.05$.

Results and Discussion

Timing of oocyte development

The ovaries of females in February 2001 contained oocytes at the early perinucleolar stages with prominent 'Balbiani body' inclusions in the ooplasm. By April 2001, during smoltification, ovaries also contained oocytes at the early periucleolar stage (see Figure 1) but several had progressed to the late perinucleolar stage where the 'Balbiani bodies' had dispersed, and one large female had ovaries containing oocytes at the very early stages of cortical alveoli accumulation. By August, females had ovaries containing oocytes at either the late perinucleolar or early cortical alveoli stage. By December all females had ovaries containing oocytes with a prominent number of cortical alveoli, and none was seen to remain at the late perinucleolar or early cortical alveoli stage. By the final sample in May 2002 many females had ovaries containing oocytes with large centrally located lipid droplets (this group of females will be designated the LD-group in subsequent text). However, there were also many females that had ovaries containing oocytes with varying degrees of cortical alveoli incorporation and no lipid droplet deposition (this group of females will be designated the CA-group in subsequent text).

Gonad growth

Total ovary mass increased with body mass from February 2001 through December 2002, with a general increase during the fall 2001, and a large increase by May 2002 (Figure 2). Gonadosomatic index (GSI), or relative gonad weight, remained constant up to October 2001 except from a transient increase in May 2001 (Figure 3). Subsequent to October, GSI increased significantly towards the end of the fall with a large increase by May 2002.

Pituitary FSH content

Pituitary FSH content increased initially up to April 2001, levels subsequently remained constant throughout the summer and increased during late fall (Figure 4). By May 2002 a large increase in pituitary FSH content had occurred. However, there was a large variation in pituitary FSH content among the females in May 2002, and this is further analyzed and discussed in a subsequent section of this report.

Plasma FSH levels

Plasma levels of FSH were close to the detection limit of the assay in August 2001 (Figure 5). A small but significant increase in plasma FSH levels occurred during the fall months, and a relatively large increase had occurred by May 2002.

Plasma 17 β -estradiol

Plasma 17 β -estradiol levels increased to initially peak during smoltification in April 2001, levels subsequently fell and then gradually increased through the summer and fall months (Figure 6). By May 2002 a large increase in plasma 17 β -estradiol levels had occurred. However, there was a large variation in plasma 17 β -estradiol levels among the females in May 2002, and this is further analyzed and discussed in a subsequent section of this report.

Ovarian gene expression

Both FSH-receptor and StAR transcript levels increased significantly from August to December 2001 (Figures 7a and b). StAR transcript levels increased between December 2001 and May 2002 while FSH-receptor did not change during this period.

Developmental groups in May 2002

By May 2002 females could be divided into two distinct groups based on the stage of oocyte development in their ovaries. Females that had ovaries containing oocytes with large centrally located lipid droplets and designated the LD-group, and those that had ovaries containing oocytes with varying degrees of cortical alveoli incorporation and no lipid droplet deposition and designated the CA-group. This difference in oocyte development was also reflected by distinct differences in physiological parameters; for example, virtually all females with low 17 β -estradiol plasma levels also had low pituitary FSH content, and these individuals belonged to the CA-group (see Figure 8).

The levels of various parameters of the CA and LD group of females were compared to analyze further the physiological differences between them in May 2002. A comparison of various parameters measured from the CA and LD groups with that of all females sampled in December, 2001 was also performed. Body weight was significantly lower for females of the CA-group compared with body weight of females December and the LD-group in May (Figure 9a). However, ovaries from the females in the CA-group in May had continued to grow, as total gonad weight for the CA-group was significantly higher than that of females in December, but lower than the LD-group in May (Figure 9b). Thus, GSI for females in the CA-group was higher than that for females in December, but lower than that for females in the LD-group during May (Figure 9c). Females in the CA-group were significantly smaller than females in the LD-group from August 2001 onwards (Figure 10). Plasma FSH levels and pituitary FSH content in all females in December and females of the CA-group in May were significantly lower than that of females of the LD-group during May (Figures 11a and b). A similar trend was seen for the FSH-receptor transcript levels in the ovary (Figure 11c). Plasma 17 β -estradiol levels were significantly lower for the females in the CA-group compared with both the females of the LD-group and females in December (Figure 12a). A similar trend was seen for the StAR transcript levels in the ovary (Figure 12b).

This study provides the first physiological evidence to support the contention that, for a fall spawning salmonid such as coho salmon, an initiation period for oocyte maturation occurs during the fall one year prior to eventual spawning. The physiological changes recorded during the fall were consistent with a role for FSH and gonadal steroids in oocyte growth during the period when oocytes are accumulating large numbers of cortical alveoli. Thus, a simultaneous rise in pituitary and plasma FSH levels, FSH-receptor gene expression in the ovary, plasma 17 β -estradiol levels, StAR gene expression occurred in the ovary, and a general increase in ovary mass occurred above that explained by the increase in body weight alone (i.e. significant increase in GSI). This is the first time these physiological parameters have been correlated with early stages of oocyte growth in any fish species, and supports the contention that the fall growth period, one year prior to spawning, has a pivotal role in controlling the initiation and maintenance of sexual maturation in salmonids.

Previous work with a variety of salmon species has related high growth in the year prior to maturation with increased rates of maturation in both sexes (see Thorpe 1986). More detailed studies in both males (see Thorpe 1994) and females (Thorpe et al. 1990, Silverstein and Shimma 1994, Duston and Saunders 1997 and 1999) have indicated that growth during the late winter or early spring in the year of maturation affects the proportion of fish maturing. It has been hypothesized that there are genetically determined thresholds of growth (e.g. size, fat level, and/or growth rate) that must be attained during these periods to allow sexual maturation to proceed (see Thorpe 1986). In addition, it has been hypothesized that some aspect of growth during the fall months (a full year prior to spawning) acts in a similar manner to control the very early stages of maturation. Thus, there is a decision period for initiation of maturation in the fall followed by a decision period (permissive) for continuing maturation in the spring (Thorpe 1994, 1998). This study provides the first evidence to associate significant physiological changes in the reproductive axis associated with these periods of proposed decision periods. It remains to be determined how growth may affect such changes in reproductive axis.

The transcription of mRNA for proteins involved in lipid and yolk deposition, as well as events at fertilization, primarily occurs during pre-vitellogenic growth (Brooks et al. 1997, Prat et al. 1998, Perazzolo et al. 1999, Kwon et al. 2001, Patino and Sullivan 2002). The regulation of such mRNA synthesis is important for the subsequent development and fertilization of oocytes. Thus, the factors controlling transcription of mRNA and production of proteins during the pre-vitellogenic period are crucial to the subsequent development of the oocytes. From the present study it is possible that FSH and gonadal steroids are involved in regulating the production of these proteins, some of which may be synthesized during the fall period. Therefore, the growth of females during this autumn period may have a significant effect on subsequent oocyte quality. Thus, growth during the year prior to spawning can have significant effects on the number, size and fertilization rate of eggs released more than 12 months in the future, as suggested in studies of salmonids by Thorpe et al. (1984), Jonsson et al. (1996), Lobon-Cervia et al. (1997), Morita et al. (1999), and Tamate and Maekawa (2000).

By the spring, a number of females appeared to have ceased or slowed oocyte development, and this was correlated with a reductions in both FSH and steroid production. Although it is not possible to say definitively that this group of females would not have spawned in November, the clear division in steroid and FSH levels between the LD and CA groups of females strongly suggests clear physiological differences between these two groups of fish. The reduced plasma levels of 17β -estradiol for the females in the CA-group compared with the females in December suggests that females in the CA-group were probably down-regulating their steroid production. In addition, previous work in coho salmon has indicated that females that are as small as those in the CA-group in May do not go on to spawn in November (Campbell et al. 2002). It is well established that 17β -estradiol regulates production of zona radiata proteins (choriogenins) and yolk proteins (vitellogenin), and fish undergoing vitellogenesis, have high levels of this steroid in their plasma (see Tyler and Sumpter 1996). Therefore, it seems clear that the females in the CA-group in the present study had made the decision not to continue ovarian growth, and this coincides with the hypothetical spring 'permissive' periods for ovarian maturation.

The destiny of the oocytes of these fish with reduced growth cannot be determined from this study. It is not known if the oocytes in the ovaries of these arrested females undergo atresia, and are then replaced the following fall by a new cohort of oocytes entering the cortical alveoli stage. As coho salmon are semelparous it is thought that, once the first phase oocyte growth is initiated, there are no oogonia left to be recruited into another cycle of oocyte growth. However, there are no experimental data to verify this. Therefore, it is not known whether semelparous fish have the capacity to replace oocytes lost to atresia. To the contrary, there is evidence to suggest that poor body growth during critical maturation decision periods may result in massive necrosis and dysfunction of the ovary, resulting in what are thought to be sterile fish(see Campbell et al. 2002). Thus, it is critical that females be maintained on a high growth rates during critical periods of early ovarian development to prevent drastic losses of oocytes that substantially reduce fecundity, and in extreme cases cause sterility.

Females in the CA-group in May 2002 had lower body growth compared to those in the LD-group from the previous August onward, and continuing through the fall and spring period. This confirms results from a previous study in which the growth histories of female coho that failed to spawn at 3-years of age were compared with females that successfully spawned at 3-years of age (Campbell et al. 2002). Histology of ovaries during the fall period indicated that all females sampled had oocytes with cortical alveoli present, thus, no females had apparently arrested at earlier stages of oocyte development. This suggests that all females undergo initial stages of oocyte maturation at 2-years of age, but that body growth affects the transition to more advanced stages. The contention is that it is the rate at which the oocytes progress through this stage of development during the fall and winter months that determines if they have reach a sufficient stage in the spring to continue up-regulating FSH and steroid production, and advance into the lipid droplet stage or even more advanced stages of oocyte development. From the body growth history data it is clear that growth during the late summer and fall months are important to ensure that ovarian development proceeds at an adequate rate so that,by the

spring, a further up-regulation of the reproductive system can proceed and ovarian maturation can continue.

Data Management Activities

Data are collected manually by NOAA, UW, and PSMFC researchers onto preformatted data sheets or directly into PCs. Data are entered and summarized on personal computers operated by researchers using Microsoft Excel, Statview 512+, Cricket Graph 1.3.2, and Prism. Data on histology of tissues is recorded using a digital camera and stored as either TIFF or JPG files on a Power MacIntosh G4. Backup copies of data are saved on an external Lacie hard-drive and on CD-ROMs. All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report under Materials and Methods. Data analyses are reported in the Results section of the annual report.

Summary and Conclusions

In conclusion, significant changes in pituitary FSH and ovarian steroid production occurred during the fall, one year prior to spawning, when oocytes were growing and cortical alveoli first appeared in the ooplasm. These data suggest that FSH plays a role in regulating oocyte growth during this period possibly by the up-regulation of ovarian steroid production. By spring, a number of females had ceased or slowed oocyte development, with a corresponding reduction in both FSH and steroid production. It is hypothesized that this group of females would not mature at age 3, but would delay maturation to age 4. Furthermore, the physiological commitment to mature was dependent on the continued up-regulation of FSH and steroid production during the spring period of pre-vitellogenic oocyte growth.

Females that ceased or slowed oocyte development in the spring had lower body growth from the previous August onward compared with females that continued oocyte growth. This indicates that growth during the late summer and fall, one year prior to spawning, can determine the decision to mature the following spring. Therefore it is important to maintain the growth of broodstock during the summer/fall period to ensure the continuation of ovary development in the subsequent spring.

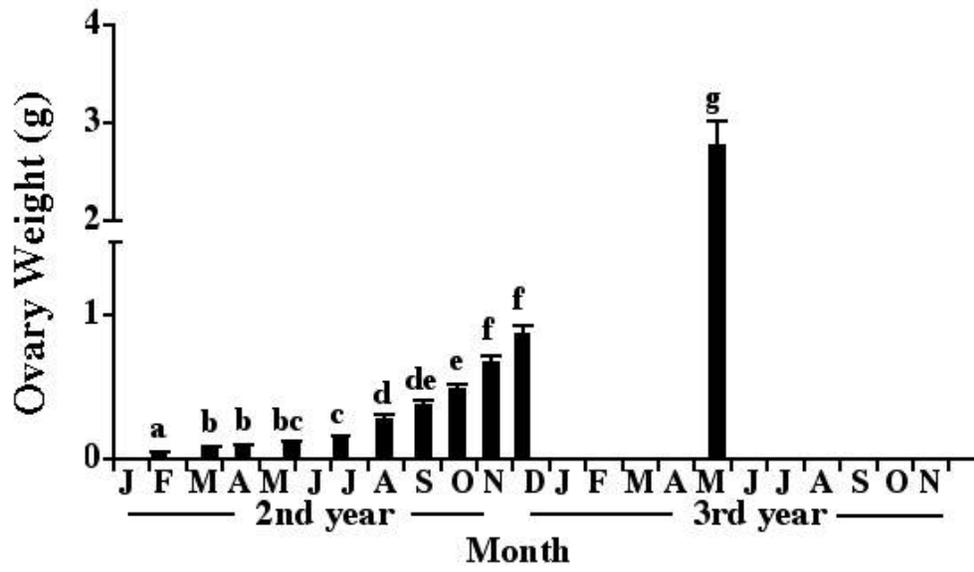


Figure 2. Change in ovary weight (g) during the rearing period. Bars represent mean \pm S.E. Bars without common superscript letter are significant differences ($P < 0.05$).

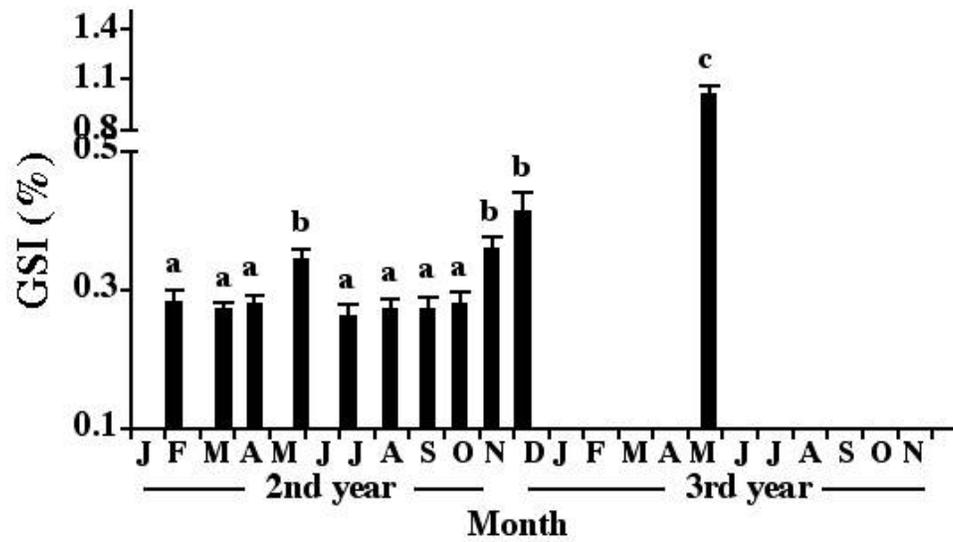


Figure 3. Change in GSI (%) during the rearing period. Bars represent mean \pm S.E. Bars without common superscript letter are significant differences ($P < 0.05$). (n=8-35)

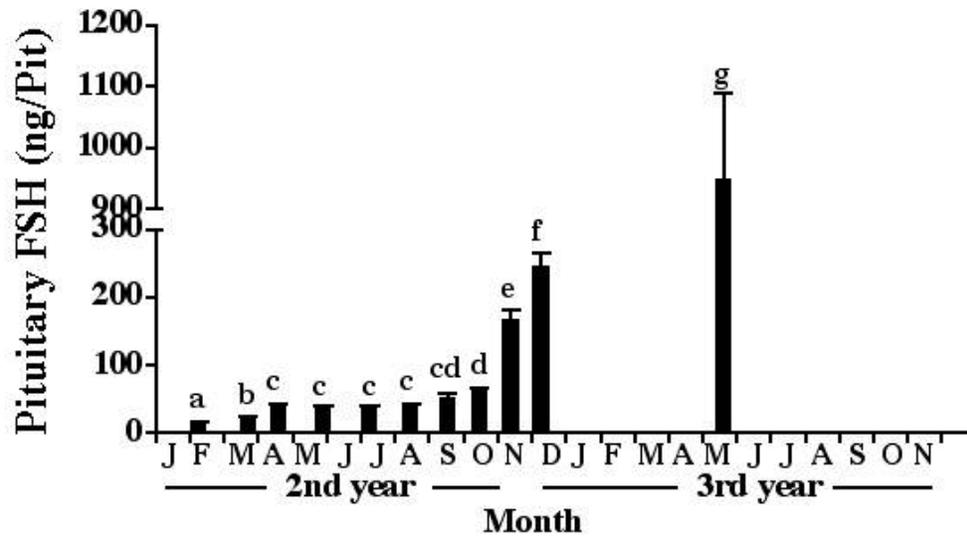


Figure 4. Change in pituitary FSH content (ng/pituitary) during the rearing period. Bars represent mean \pm S.E. Bars without common superscript letter are significant differences ($P < 0.05$). (n=8-35)

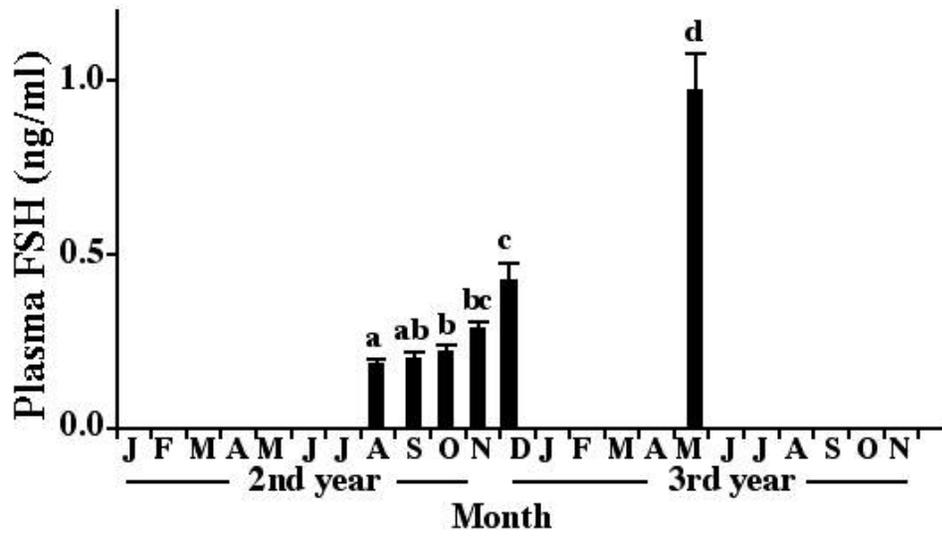


Figure 5. Change in plasma FSH (ng/ml) during the rearing period. Bars represent mean \pm S.E. Bars without common superscript letter are significant differences ($P < 0.05$). (n=14-35)

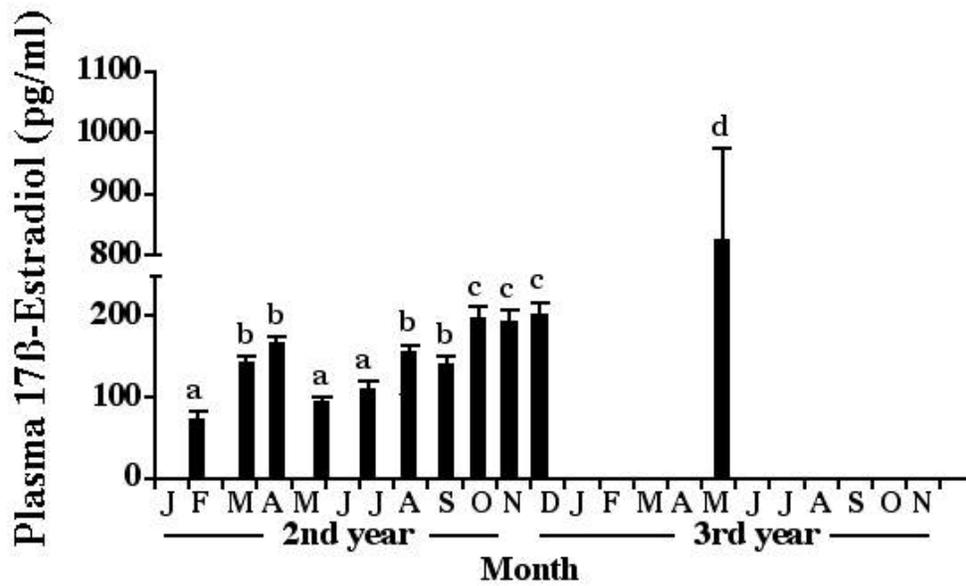


Figure 6. Change in plasma 17β-estradiol (pg/ml) during the rearing period. Bars represent mean ± S.E. Bars without common superscript letter are significant differences (P < 0.05). (n=8-35)

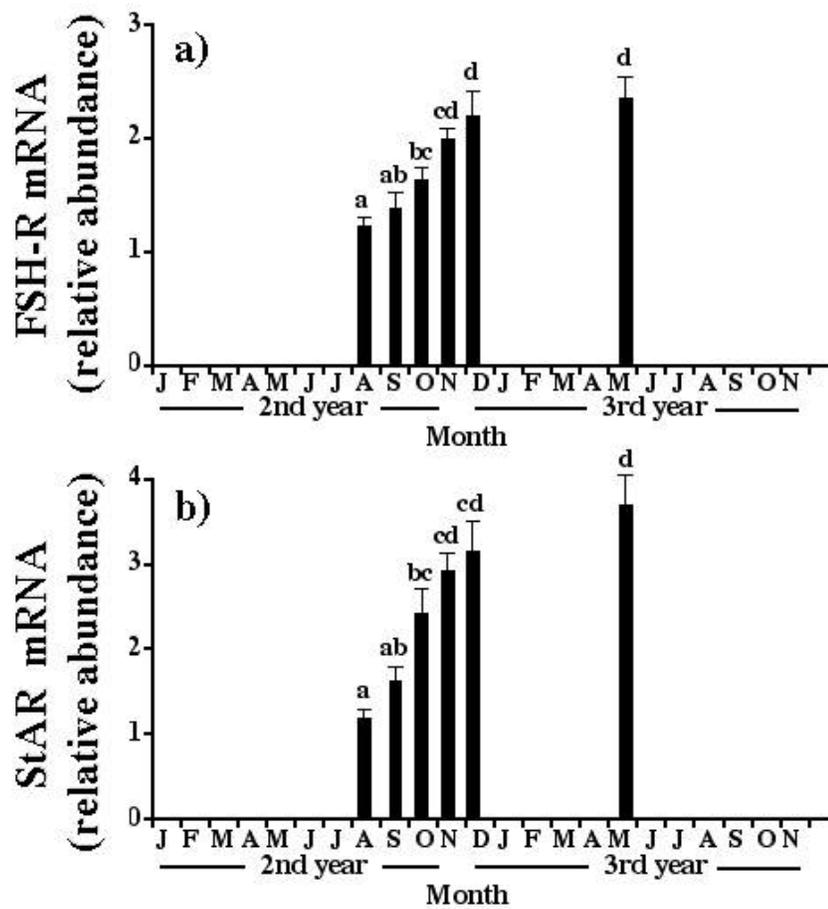


Figure 7. Change in relative abundance of a) FSH-R mRNA and b) StAR mRNA in ovary tissue during the fall and final spring rearing period. Bars represent mean \pm S.E. Bars without common superscript letter are significant differences ($P < 0.05$). (n=14-35)

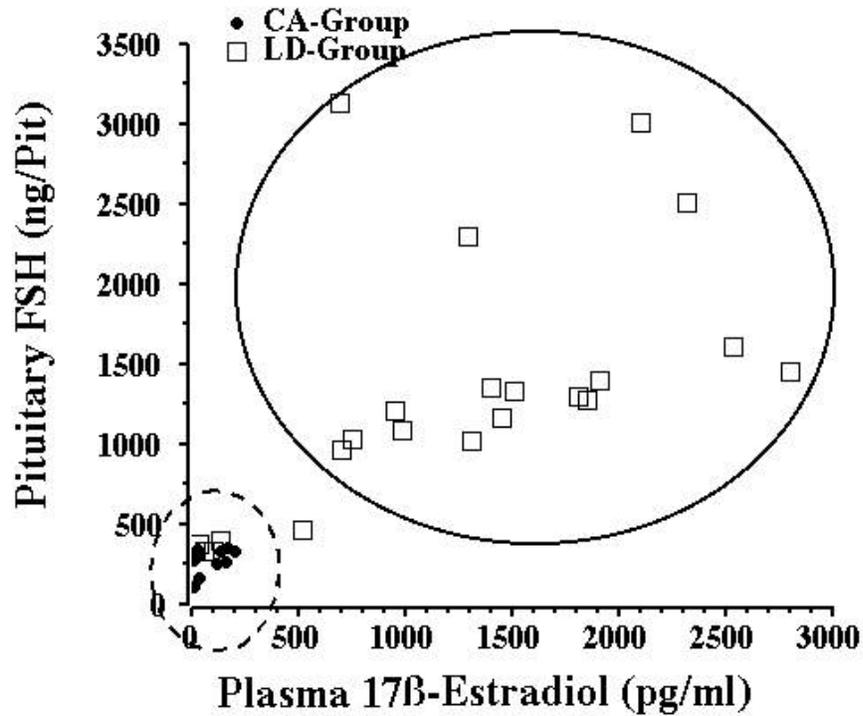


Figure 8. Plot of plasma 17β-estradiol versus pituitary FSH content for the females sampled in May 2002. The circles indicate two distinct groups of females. Solid circle indicates the group that had both high plasma 17β-estradiol and pituitary FSH and this group coincided with females that had ovaries containing oocytes at the advanced stage of lipid droplet incorporation (LD). Dashed circle indicates the group which had both low plasma 17β-estradiol and pituitary FSH and this group coincided with females that had ovaries containing oocytes at the less advanced stage of cortical alveoli incorporation (CA).

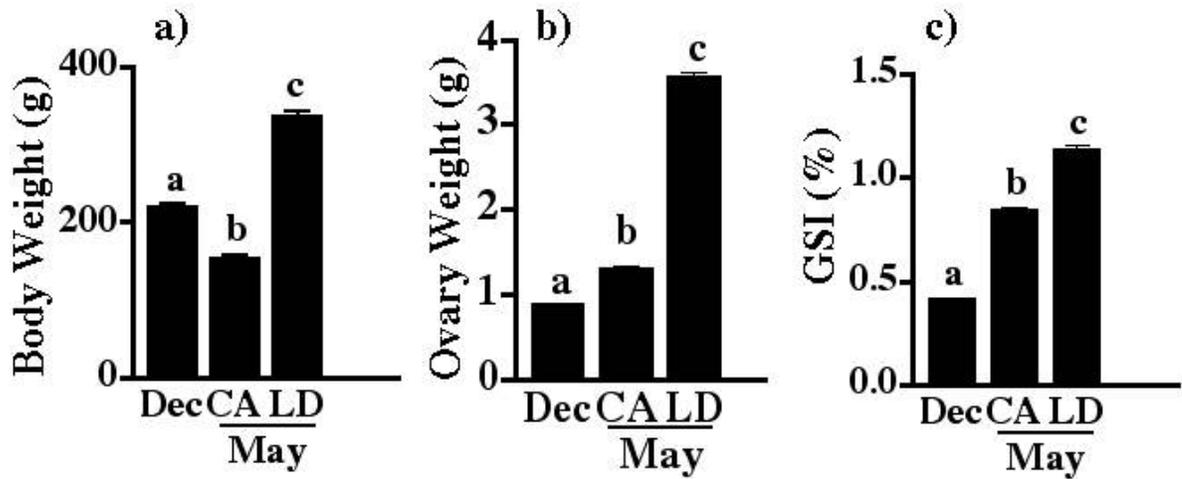


Figure 9. Average body weight (a), ovary weight (b) and GSI (c) for the LD and CA groups of females in May 2002 and females in December 2001. Bars represent mean \pm S.E. Bars without common superscript letter are significant differences ($P < 0.05$). LD-n=21, CA-n=12, Dec-n=14.

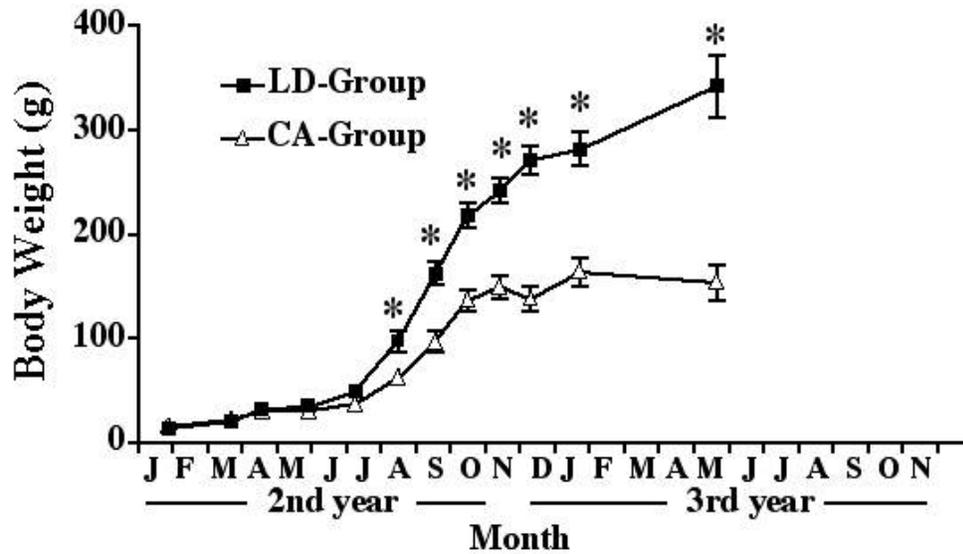


Figure 10. Growth history for the females sampled in May 2002. The LD-group corresponds to females that had ovaries containing oocytes at the advanced stage of lipid droplet incorporation, and the CA-group corresponds to females that had ovaries containing oocytes at the less advanced stage of cortical alveoli incorporation. Points represent mean \pm S.E. of body weight (g). The stars indicate significant difference between the two groups within the same sample date ($P < 0.05$). LD-n=21, CA-n=12.

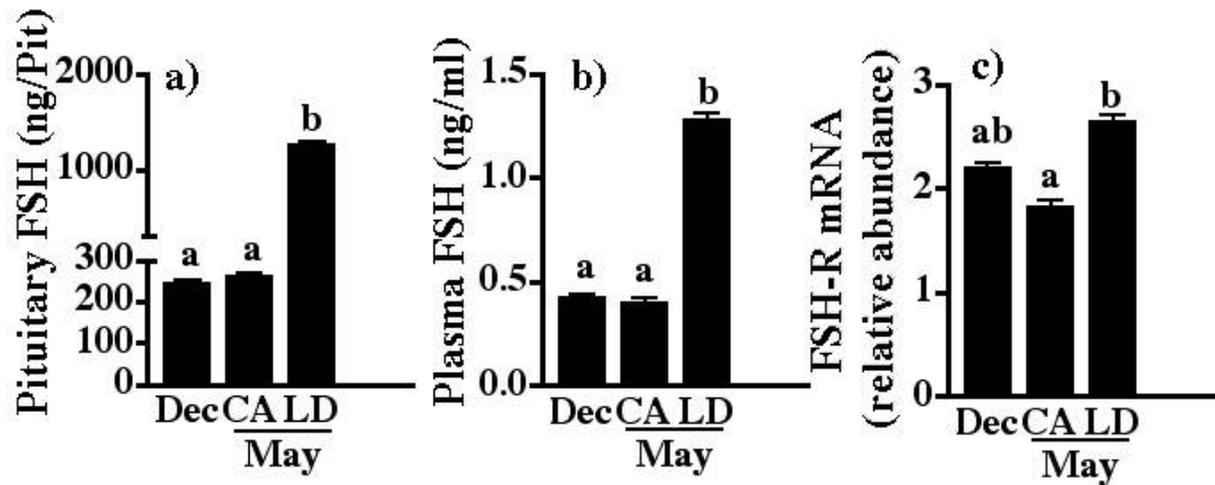


Figure 11. Average pituitary FSH content (a), plasma FSH (b) ovary FSH-receptor mRNA relative abundance (c) for the LD and CA group of females in May 2002 and females in December 2001. Bars represent mean \pm S.E. Bars without common superscript letter are significant differences ($P < 0.05$). LD-n=21, CA-n=12, Dec-n=14.

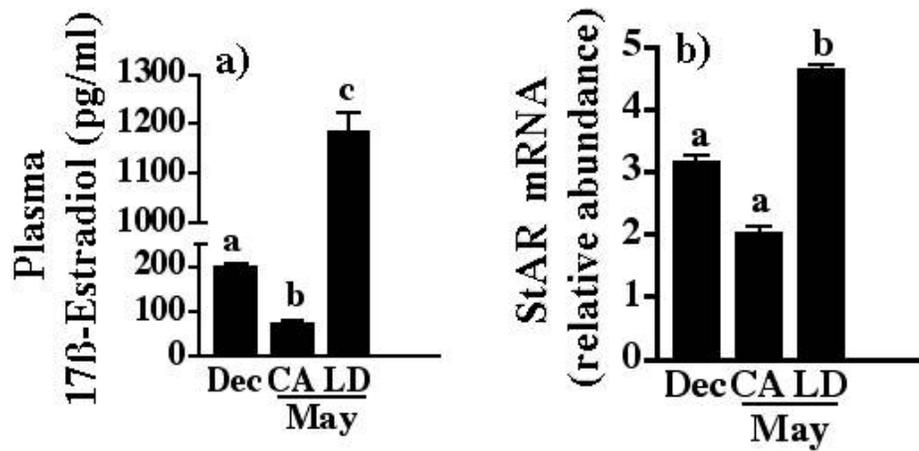


Figure 12. Average plasma 17β-estradiol (a) ovary StAR mRNA relative abundance (b) for the LD and CA group of females in May 2002 and females in December 2001. Bars represent mean ± S.E. Bars without common superscript letter are significant differences (P < 0.05). LD-n=21, CA-n=12, Dec-n=14.

References

- Beacham, T.D., and C.B. Murray. 1993. Fecundity and egg size variation in North American Pacific salmon *Oncorhynchus*. *J. Fish Biol.* 42:485-508.
- Brooks, S., C.R. Tyler, and J.P. Sumpter. 1997. Egg quality in fish: what makes a good egg? *Rev. Fish Biol. Fish.* 7:387-416.
- Bromage, N., and R. Cumaranatunga. 1988. Egg production in the rainbow trout. *In* J.F. Muir and R.J. Roberts, (editors), *Recent advances in aquaculture* (Vol. 3, p. 63-138. Croom Helm, London.
- Campbell, B., B.R. Beckman, W. Fairgrieve, J.T. Dickey, and P. Swanson. 2002. Effects of growth on age of maturation, fecundity and egg size in female coho salmon. *In* Berejikian, B.A. *Research On Captive Broodstock Programs For Pacific Salmon*. Report to BPA Contract No. 0000522-7, Report DOE/BP-0005227-2. pp 36-61.
- Du, S.J., R.H. Devlin, and C.L. Hew. 1993. Genomic structure of growth hormone genes in chinook salmon (*Oncorhynchus tshawytscha*): presence of two functional genes, GH-I and GH-II, and a male-specific pseudogene, GH-psi. *DNA Cell Biol* 12:739-51.
- Duston, J., and R.L. Saunders. 1997. Life histories of Atlantic salmon altered by winter temperature and summer rearing in fresh- or sea-water. *Environ. Biol. Fish.* 50:149-166.
- Duston, J., and R.L. Saunders. 1999. Effect of winter food deprivation on growth and sexual maturity of Atlantic salmon (*Salmo salar*) in seawater. *Can. J. Fish. Aquat. Sci.* 56:201-207.
- Fleming, I.A., and M.R. Gross. 1990. Latitudinal clines: A trade-off between egg number and size in pacific salmon. *Ecology* 71:1-11.
- Fleming, I.A., A. Lamberg, and B. Jonsson. 1996. Effects of early experience on the reproductive performance of Atlantic salmon. *Behav. Ecol.* 8:470-480.
- Groot, C., and L. Margolis. 1991. *Pacific salmon life histories*. UBC Press, Vancouver, 564 p.
- Healey, M.C., and W.R. Heard. 1984. Inter- and intra- population variation in the fecundity of chinook salmon (*Oncorhynchus tshawytscha*) and its relevance to life history theory. *Can. Fish. Aquat. Sci.* 41:476-483.
- Hutchings, J.A. 1991. Fitness consequences of variation in egg size and food abundance in brook trout *Salvelinus fontinalis*. *Evolution* 45:1162-1168.
- Jonsson, N., and B. Jonsson. 1999. Trade off between egg mass and egg number in brown trout. *J. Fish. Biol.* 55:767-783.
- Jonsson, N., B. Jonsson and I.A. Fleming. 1996. Does early growth cause a phenotypically plastic response in egg production of Atlantic salmon. *Func. Ecol.* 10:89-96.
- Khoo K.H. 1979. The histochemistry and endocrine control of vitellogenesis in goldfish ovaries. *Can. J. Zool.* 57:617-626.
- Kwon J.Y., F. Prat, C. Randall, and C.R. Tyler. 2001. Molecular characterization of putative yolk processing enzymes and their expression during oogenesis and

- embryogenesis in rainbow trout *Oncorhynchus mykiss*. Biol Reprod. 65:1701-1709.
- Lobon-Cervia, J., C.G. Utrilla, P.A. Rincon, and F. Amezcua. 1997. Environmentally induced spatio-temporal variations in the fecundity of brown trout, *Salmo trutta* L.: trade-offs between egg size and fecundity. Freshw. Biol. 38:277-288.
- Miller, W.L. and J.F. Strauss III. 1999. Molecular pathology and mechanism of action of the steroidogenic acute regulatory protein, StAR. J. Steroid Biochem. Mol. Biol. 69:131-141.
- Morita, K., S. Yamamoto, Y. Takashima, T. Matsuishi, Y. Kanno and K. Nishimura. 1999. Effect of maternal growth history on egg number and size in wild whitespotted charr (*Salvelinus leucomaenis*). Can. J. Fish. Aquat. Sci. 56:1585-1589.
- Patino, R., and C.V. Sullivan. 2002. ovarian follicle growth, maturation, and ovulation in teleost fish. Fish Physiol. Biochem. 26:57-70.
- Perazzolo, L.M., K. Coward, B. Davail, E. Normand, C.R. Tyler, F. Pakdel, W.J. Schneider and F. Le Menn. 1999. Expression and localization of messenger ribonucleic acid for the vitellogenin receptor in ovarian follicles throughout oogenesis in the rainbow trout, *Oncorhynchus mykiss*. Biol. Reprod. 60:1057-1068.
- Piper, R.G., I.B. McElwain, L.E. Orme, J.P. McCraren, L.G. Fowler, and J.R. Leonard. 1983. Fish hatchery management. Am. Fish. Soc., Bethesda, MD, 537 p.
- Prat, F., K. Coward, J.P. Sumpter, and C.R. Tyler. 1998. Molecular characterization and expression of two ovarian lipoprotein receptors in the rainbow trout, *Oncorhynchus mykiss*. Biol. Reprod. 58:1146-1153.
- Roff, D.A. 1992. The evolution of life histories. Chapman and Hall, New York, 535 p.
- Shearer K.D., and P. Swanson. 2000. The effect of whole body lipid on early sexual maturation of 1+ age male chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture 190:343-367.
- Silverstein, J.T. and H. Shimma. 1994. Effect of restricted feeding on early maturation in female and male amago salmon, *Oncorhynchus masou ishikawae*. J. Fish Biol. 45:1133-1135.
- Stearns, S.C. 1992. The evolution of life histories. Oxford Univ. Press, Oxford, 249 p.
- Tamate, T., and K. Maekawa. 2000. Interpopulation variation in reproductive traits of female masou salmon, *Oncorhynchus masou*. Oikos 90:209-218.
- Thorpe, J.E. 1986. Age at first maturity in Atlantic salmon, *Salmo salar*: Freshwater period influences and conflicts with smolting. In D.J. Meerburg (editor), Salmonid age at maturity. Can. Spec. Publ. Fish. Aquat. Sci. No. 89. pp. 7-14.
- Thorpe, J.E. 1994. Reproductive strategies in Atlantic salmon, *Salmo salar* L. Aquacult. Fish. Manage. 25:77-87.
- Thorpe, J.E., M.S. Mile and D.S. Keay. 1984. Developmental rate, fecundity and egg size in Atlantic salmon, *Salmo salar*. Aquaculture 43:289-305.
- Thorpe, J.E., C., Talbot, M.S. Mile and D.S. Keay. 1990. Control of maturation in cultured Atlantic salmon, *Salmo salar*, in pumped seawater tanks, by restricting food intake. Aquaculture 86:315-326.
- Thorpe, J.E., M. Mangel, N.B. Metcalfe and F.A. Huntingford. 1998. Modelling the proximate basis of salmonid life-history variation, with application to Atlantic

salmon, *Salmo salar* L. Evol. Ecol. 12:581-599.
Tyler, C.R. and J.P. Sumpter. 1996. Oocyte growth and development in teleosts. Rev.
Fish Biol. Fish. 6:287-318.

OBJECTIVE 3 - IMPROVE PHYSIOLOGICAL DEVELOPMENT AND MATURATION

TASK 6. MONITORING REPRODUCTIVE DEVELOPMENT IN CAPTIVE BROODSTOCK AND ANADROMOUS HATCHERY STOCKS OF SNAKE RIVER SPRING CHINOOK SALMON DURING THE FRESHWATER PHASE OF ADULT MIGRATION

By

Penny Swanson, Briony Campbell, Brian R. Beckman, Donald A. Larsen, Jon T. Dickey, Kathy A. Cooper, Nicholas Hodges, Paul Kline, Karl D. Shearer, David Venditti, Gary A. Winans, and Graham Young

Introduction

Delays in spawning time of captively-reared Pacific salmon relative to wild fish of the same stocks are not uncommon. Varying degrees of delayed spawning have been observed in several captive broodstock programs including those for the Oregon and Idaho stocks of Snake River spring chinook salmon, Redfish Lake sockeye salmon, and Sacramento River winter run chinook salmon.

The degree of the spawning delay varies with the stock and even year to year within the same stock of fish. Delays in spawn time have ranged from 2-6 weeks. This does not pose serious limitations for the captive broodstock programs when gamete quality has been acceptable and only mating between captively-reared adults is performed. However, it can be problematic if the delay in spawning time also reduces gamete quality and if mating of captive fish with wild fish is desired. In a captive rearing strategy, such as the one employed by the Idaho Fish and Game Department (IDFG) Spring Chinook Salmon Captive Broodstock Program, adult fish are released into their native streams to spawn with wild counterparts. This strategy is presently limited because the seasonal timing of ovulation and spermiation in some of the captive populations has been 3-5 weeks later than wild counterparts (P. Kline, IDFG, personal communication), making spawning of captively-reared adults with wild fish improbable. Similarly, in the Sacramento River Winter Run Chinook Captive Broodstock Program, gametes from captive broodstock are utilized when insufficient numbers of gametes are available from wild returning adults. In this program, the captive adults have delayed spawning time relative to the wild fish, and photoperiod manipulations have been utilized to advance spawn time of captive adults (K. Arkoosh, Bodega Marine Laboratory, University of California, personal communication).

In addition to the delay in spawn time, captively-reared females can also display a high rate of egg retention and, in some cases, abnormal ovarian development leading to

reduced fertility, egg size and number, and atretic eggs. In concert with the delay in spawning time, a progressive decline in egg quality has been observed: female Redfish Lake sockeye salmon captive broodstock spawned in the middle of the spawning season produce offspring with higher survival to the eyed stage than females spawned late in the season (C. McAuley, NMFS, personal communication). The factors causing delayed maturation are not known, but probably it is due to rearing environment, as genetic selection for spawn time has not occurred in the captive broodstock programs that were established as part of recovery for endangered or threatened stocks of Pacific salmon.

While photoperiod is a major cue for seasonal timing of maturation in salmonids, the captive broodstock are generally reared under natural photoperiod. Thus, photoperiod cannot explain the differences in spawn time between wild and captive fish. The seasonal delay in ovulation and asynchronous maturation of oocytes within the ovary may be a manifestation of abnormalities in the rate of oocyte development due to inappropriate environmental cues, such as temperature, and/or growth conditions. Abnormal ovarian development may be due to rearing conditions at various times, for example, early in the life cycle, during the final year prior to spawning when secondary oocyte growth is occurring, and/or during the final stages of oocyte maturation prior to ovulation.

In order to develop rearing conditions that allow for proper seasonal timing of oocyte growth and maturation, a better understanding of the phase of reproduction during which the delay first occurs is needed. In other words, is the delayed development of the egg occurring during yolk incorporation (seawater rearing) or just during the final phase of oocyte maturation and ovulation (freshwater rearing prior to spawning)? By comparing reproductive development of spring chinook captive broodstock with that of a closely related stock of migrating hatchery fish, it may be possible to determine at what stage the captively-reared fish are delayed, and to develop a rational approach to solving this problem.

In the present study, reproductive development was compared in two stocks of Snake River spring chinook salmon: (i) adults returning to the IDFG Rapid River Hatchery during spring through summer of 2001 and, (ii) brood year 1997 Lemhi River captive broodstock reared at the NMFS Manchester Research Station and the IDFG Eagle Fish Hatchery. The initial intent of the study was to characterize the process of sexual maturation in Snake River spring chinook salmon during the upstream migration. However, obtaining samples from fish during the migration was not possible since endangered and non-endangered stocks could not be distinguished at the sampling locations along the migratory corridor. Therefore, the goals of the study were to:

- compare the progress of sexual maturation by histological analyses of gonad samples and measurement of reproductive hormones,
- evaluate the bioenergetics associated with maturation in returning adult hatchery fish,
- determine the morphological changes that occur during sexual maturation in spring chinook salmon.

Concurrent with the hormonal changes that occur in sexually maturing salmon as they ascend the river is a depletion of body stores of protein and lipid. This depletion occurs due to cessation of feeding after entering fresh water, energy expenditure during upstream migration and transfer of somatic nutrients to the gonads. The relationships among changes in hormones, body morphology, body composition, and gonad development have not been well documented in migrating adult chinook salmon. Not surprisingly, anecdotal information suggests that the compositional changes that occur in the body of captive broodstock are far less than that of wild fish that undergo an upriver migration. The large returns of adult spring chinook salmon during 2001 provided a unique opportunity to obtain important physiological information from adult spring chinook salmon during the 4-5 months preceding spawning. The initial intent of the study was to characterize the process of sexual maturation in Snake River spring chinook salmon during the upstream migration. However, obtaining samples from fish during the migration was not possible as endangered and non-endangered stocks could not be distinguished at the sampling locations along the migratory corridor. Therefore, reproductive physiology of fish returning to the IDFG Rapid River Hatchery was characterized from the period when adults first appeared in Rapid River (May) through spawning in August and September. These data provide a template by which to compare captively-reared adult spring chinook salmon, and information important to aid understanding the underlying causes of the delayed maturation in the captive broodstock.

In addition to generating basic comparative information on the reproductive physiology of migrating and captive spring chinook salmon, a preliminary experiment was conducted to determine if temperature upon transfer of adults to fresh water in the spring affected the timing of spawning or egg quality in the subsequent fall. This experiment was based on results with Atlantic salmon by Taranger et al. (1999), who demonstrated an advancement of spawning by rearing adults in chilled fresh water during the later stages of sexual maturation, and studies with rainbow trout by Billard et al. (1977), who showed that high water temperatures can delay spermiation and suppress gonadotropin levels in males.

Materials, Methods, and Description of Study Area

Experimental design

The process of sexual maturation in male and female Rapid River chinook salmon returning to the IDFG Rapid River Fish Hatchery (Riggins, Idaho) was compared with that of captively-reared Lemhi River spring chinook salmon females from May through September 2001. The effects of temperature at the time of fresh water transfer on maturation were tested in the Lemhi River females. Lemhi River captive broodstock (broodyear 1997) were reared in filtered and UV-treated saltwater at the Manchester Research Station (MRS) from May 1999 through early June 2001. On June 8, 2001 all maturing fish were transferred to fresh water and reared through spawning at the IDFG Eagle Hatchery, Eagle, ID. At that time, Lemhi River captive broodstock were divided into two groups: chilled (9°C) and ambient (14°C). Fish were maintained in the treatment

groups until spawning in the hatchery in September and early October 2001. Fish were not fed during the course of this study. However, prior to May 2001, fish were fed a Smolt HP from 0-5 months after transfer to seawater as 1+ age smolts, Nutrafry from 5-8 months post seawater transfer, and Salmon Pedigree Diet supplemented with 100 ppm astaxanthin from 8 months to maturity. All diets were purchased from Moore Clark. Food was withdrawn from Lemhi River females the first sampling in May 2001, and fish were not fed subsequently.

Sampling protocol

Samples of gonads, blood, pituitary glands and carcasses were collected from adults that returned to the IDFG Rapid River Fish Hatchery at monthly intervals from May through spawning in September 2001. A total of 10 males and 10 females were sacrificed each month. Photographs were taken of whole bodies and gonads using a digital camera. At spawning, mean egg weight was determined by weighing a sample of 50 ovulated eggs. Fecundity was determined by weighing all ovulated eggs and dividing by mean egg weight. Similar samples were collected during 2001 from female (BY97) Lemhi River spring chinook salmon captive broodstock at three time points: May (just prior to transfer from the saltwater rearing tanks to fresh water, n= 8 females, 1 male), August (prior to the normal time of release of adults into the Lemhi River for spawning; n= 8-9 per temperature treatment), and September/October (when fish were spawned in the hatchery, n=5 per temperature treatment). Because of logistical difficulties, it was not possible to photograph captive broodstock adults at the time of spawning in the hatchery; however, all other samples were collected at this time. Spawn times were also compared with Lemhi River captive broodstock (BY 97 and BY 96) that were reared completely in fresh water at the IDFG Eagle Hatchery. No samples were collected from these fish during the rearing period.

During the sampling of Lemhi River captive broodstock in May 2001, substantial gastric distention and ovarian atresia, particularly of the left ovary was observed. To determine whether this was specific to the Lemhi River stock, or due to the rearing in saltwater at the MRS, 5 females from the BY 97 Catherine Creek spring chinook salmon captive broodstock maintained in fresh water at the Bonneville Hatchery, and 5 females of the same stock and year class reared in saltwater at the MRS were sampled on May 21 and 22, 2001. A schematic of the various rearing environments and sampling times is shown in Figure 1.

Gonad morphology and histology

All gonads were removed, weighed, and photographed with a digital camera. Pieces of testicular tissue were preserved in Bouin's fixative for 48 hours at 4°C and transferred to 70% ethanol for storage. Tissue was processed and embedded in paraffin. Sections were cut at 4 microns on a standard rotary microtome, and stained with routine hematoxylin and eosin. Pieces of ovarian tissue was preserved in Karnovsky's fixative for 48 hours at 4°C and transferred to 70% ethanol for storage. Tissue was processed and embedded in glycol methacrylate resin using the Technovit 7100® Kit. Sections were cut at 4 microns

on an automated microtome (Leica RM 2165) and stained with a modified hematoxylin and eosin procedure. Pieces of tissue from the anterior, middle, and posterior regions of the gonads were collected. Histology data presented in this report are only from the mid-region of the right gonad. In the Lemhi River females, which exhibited varying degrees of ovarian atresia, histology of only the healthy appearing oocytes are included in this report as the objective was to compare reproductive stage and not document in detail any pathological processes. Ovaries were staged according to Naghama et al. (1983). Because all samples were in various stages of secondary oocyte growth, they were classified into primary yolk globule stage (yolk first appear, most the cytoplasm contains cortical aveoli and lipid droplets, early and late secondary yolk globule stage (cortical aveoli present only in the periphery of the cytoplasm), and early and late tertiary yolk globule stage (cytoplasm contains mostly yolk and few lipid droplets). Stages of spermatogenesis were determined by light microscopy using Loir [21] and Schulz [17] as a guide. For quantification of germ cell types, three fields of view were selected at random from a section taken from the middle portion of the testis. Using an eyepiece with a 10x10 grid, each germ cell type that fell on an intersection was identified and counted. The proportion of each germ cell type was expressed as a percentage of the total number of germ cells counted. Germ cell staging was as follows: primary A spermatogonia, transitional spermatogonia, late (secondary) B spermatogonia, primary spermatocyte, secondary spermatocyte, spermatid, and spermatozoa.

Plasma and pituitary hormone analyses

Fish were anesthetized in buffered tricaine methanesulfonate (MS-222, 0.05%) and blood was collected into 10 cc heparinized syringes from the caudal vein using 18 gauge needles. Blood was immediately transferred to 15 ml polypropylene tubes containing aprotinin and trypsin inhibitor, and stored on ice prior to centrifugation at 800g for 15 minutes. Plasma was stored on dry ice until transferred to a -70°C freezer for long-term storage. Fish were decapitated, pituitary glands were removed and frozen immediately on dry ice prior storing at -70°C until analysis for gonadotropin content. Plasma and pituitaries were analyzed for follicle-stimulating hormone (FSH) and luteinizing hormone (LH) content by radioimmunoassays (RIAs; Swanson et al. 1989). Levels of testosterone (T), 11-ketotestosterone (11-KT), and estradiol- 17β (E2), and $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ($17,20\beta$ -P) were determined in plasma samples by either RIAs or enzyme immunoassays (EIA) (Scott et al. 1982, Sower and Schreck 1982, Cuisset et al. 1994, Rodriguez et al. 2001). Plasma insulin-like growth factor (IGF-I) was measured by RIA using commercially available components (Grop Pep, Inc. Adelaide, Australia), as described by Shimizu et al. (2000).

Body composition

Carcasses, gonads, and viscera were collected separately and stored at -20°C until analyzed for fat and protein content. Gonads, viscera or carcasses were partially thawed, ground in a food processor and a sub-sample of 100g of wet material was dried, then reground in a coffee grinder, and a sub sample was taken for analysis (0.5g for protein and 2g for fat). Moisture was determined by drying to constant weight at 105°C . Fat was

determined using a Soxhlet device (Buchi 810, Brinkman Instruments, Westbury, NY) with dichloromethane as the solvent. Protein was calculated by multiplying percent nitrogen determined using a nitrogen analyzer (Leco FP2000, Leco Corp., Henderson, NV) by a factor of 6.25. Proximate composition values are expressed on a wet weight basis.

Morphometric analyses

Fish were euthanized and photographed with a digital camera on their left side adjacent to a ruler. Cartesian coordinate information for 19 landmarks was collected from each image with the digitizing program *tpsDig* (Rohlf 1998). Thirty-two distance measurements were calculated between 15 landmarks in a truss network pattern (see Figure 2, after Winans 1984). Landmarks at the anterior tip of the dorsal and anal fins, and at the insertion and distal point of the left pectoral fin were digitized for dorsal and anal fin height, and pectoral fin length, respectively. Distances were calculated using the Pythagorean theorem. Two analyses, principal component (PC) and relative warp (RW) analyses (Cadrin 2000) were conducted to assess multivariate shape variation. Morphometric distance data were analyzed in a PC analysis of the variance-covariance matrix. RW analyses, representing geometric-landmark methods (Bookstein 1991), were conducted using *TPSRELW* (Rohlf 1998) to assess the geometry of each fish relative to an average or consensus body shape. Individual PC and RW scores were analyzed and plotted for the first major components, respectfully. Bivariate confidence ellipses were calculated from standard deviations and sample covariation.

Results

Size and composition

There were no significant changes in length in the Rapid River males and females over time, but body weight declined over the course of the sampling period (Figure 3). Lemhi River females were approximately 50% lower in weight and 33% less in length than the Rapid River females. Body weight and length of the Lemhi River females sampled in September were significantly lower than the fish sampled during May and August.

Carcass fat content (Figure 4) and visceral fat content (Figure 5a) declined over time in both the Rapid River and Lemhi River fish; however, the Lemhi River females had higher body and visceral fat content than the Rapid River females at all time points. In August and at spawning, the Lemhi River females maintained on chilled water had significantly higher body fat content than the fish reared on ambient water temperature. At spawning, visceral fat content was significantly higher in Lemhi River females reared in chilled water compared with those reared in ambient water, but no differences were observed in the previous August sampling. In the Rapid River females, ovarian fat content declined approximately 50% from May to spawning in September (Figure 5b). In May and August, the mean ovarian fat content of Lemhi River females was significantly lower than that of the Rapid River females and did not decline in August as was observed

for the Rapid River females. However, the mean fat content of ovulated eggs collected at spawning was similar between the two groups and not affected by rearing temperature for the Lemhi females. In contrast to the ovaries, the fat content of the testes in Rapid River males increased approximately 2-fold over time (Figure 5b).

Carcass protein (Figure 6) declined over time in all groups of fish with the greatest decline occurring from August to spawning. At the time of spawning, the carcasses of Lemhi River females reared on chilled water had higher protein content than that of the fish reared on ambient water. Visceral protein declined from July to September (spawning) in both male and female Rapid River spring chinook salmon (Figure 7a). The decline in visceral protein in the Lemhi River females from August to spawning was more substantial and levels at spawning were significantly lower than Rapid River females. Visceral protein content in the Lemhi River females in May was significantly lower than that of the Rapid River females, and levels increased in August. Gonad protein content increased over time in both sexes of Rapid River fish, but the increase in males was greater than that of females. Ovarian protein content in the Rapid River fish increased approximately 12%, while that of the testes increased 60%. In the Lemhi River females, ovarian protein was significantly lower than that of Rapid River females at all time points. There was no effect of rearing temperature on protein content of ovaries collected in August or ovulated eggs at spawning.

Gross morphology

The external morphology and gross morphology of the gonads of Rapid River females, Lemhi River females, and Rapid River males are shown in Figures 8-10. Photographs are of individuals that were representative of the fish sampled at each time point. Data on gonad morphology and histology from all Lemhi and Catherine Creek females sampled are shown in Appendix 1. Examples of the ovarian atresia associated with the gastric distention disorder that were observed in the Lemhi River broodstock reared in seawater at MRS are shown in Figure 9. The ovarian atresia occurred primarily in the left ovary, which was often twisted and wrapped around the stomach. Adhesions to the stomach were observed in fish with severely twisted and hemorrhaging ovaries. Similar ovarian regression was observed in the 4 out of 5 fish sampled in May 2001 from the Catherine Creek spring chinook salmon reared in seawater at MRS, but not in any of the 5 fish sampled in May 2001 from the parallel groups reared in fresh water at the Bonneville Hatchery (Appendix 1).

Morphometric analyses

For the Rapid River chinook salmon, fin lengths were greater in males than females in each sampling period (Figure 11). For females, mean fin sizes were smaller in May and August; otherwise there were no trends in fin size. For males, median fins (dorsal and anal) did not change significantly from May to September. Pectoral fin lengths increased in size in August and September, but were not statistically significant from previous sample dates. Pelvic fin size did not change for males from May to August (mean values of 74-79mm), but increased significantly in September (mean pelvic fin length 90mm).

Gender-specific tail shape differences as described for mature sockeye salmon (Winans et al. 2003) were not found in the Rapid River spring chinook salmon.

The first three principal components explained 93.6% of the overall variance. PC1 explained 86.6% of the variance and was a size component. PC1 scores were positively correlated to fork length ($r = 0.99$) and body weight ($r = 0.94$). No other components were significantly correlated with these two univariate size variables. PC2 explained 5.3% of the variance and identified sex-related shape differences that varied systematically (i.e., ontogenetically) through the sampling period (Figure 12). There was a significant difference between males and females at PC2 that increased from May ($t_{(d.f.=16)} = 2.27, P = 0.043$) to September ($t_{(d.f.=16)} = 10.13, P = 0.001$). For males, PC2 values increased throughout the sampling periods, with a significant increase in September (Figure 12). For females, PC2 values also generally increased through time, although there was a dip in values in August and July and September values were approximately equal (Figure 12). Important morphometric characters identified by PC2 are illustrated in Figure 13. In general, an increase in PC2 was associated with an increase in head size and a proportional decrease in trunk measures. Two specific morphometric changes associated with PC2, D1-14, and D1-15 are illustrated in Figures 14 and 15, respectively.

Similar but less pronounced ontogenetic shape differences were seen in the warp analysis. Along relative warp 2 (RW2), males differentiated from females in August, a difference that increased in the September collections (Figure 16). The geometry of the males (i.e., negative RW2 values) compared with the consensus body form is illustrated in Figure 17. The changes associated with RW2 involve the lower head and the tail, similar to PC2.

Females from Lemhi River (May and August) and Catherine Creek (May only; approx. 20 fish each) spring chinook salmon captive broodstock were smaller than the Rapid River samples. No comparisons of fin sizes were made. In a principal component analysis, the three collections had intermediate values of PC2 in comparison to the Rapid River time series (Figure 18). Although statistically significant differences were noted at PC3 – PC5, no discernible ontogenetic patterns were seen (results not shown).

Gonad development

Gonadosomatic indices, or GSI, [(gonad weight/body weight) x 100], increased in both Lemhi and Rapid River females over time; however, Lemhi River females had lower GSI in August than the Rapid River females (Figure 19). The lower GSIs in Lemhi River females could be in part due to the widespread atresia, primarily in the left ovary that was observed in many of the females and the relative state of immaturity of the oocytes of Lemhi River females compared with that of the Rapid River fish. Histological examination of the ovaries of the Rapid River females indicated that, by the first sampling in early May, oocytes were already in the secondary yolk globule stage and progressed to the tertiary yolk globule and migrating nucleus stages by August. This was accompanied by increases in egg diameter, although this parameter was not quantified

because of some distortions due to fixation. Among the Rapid River females, the stage of oocyte development was consistent among individuals sampled on a given date. In contrast, the Lemhi River females were slightly less advanced and more variable in stage of maturation on a given date than the Rapid River females. Examples of oocyte stages observed in May and August are shown in Figure 19. In May Lemhi River females were either in the primary or secondary yolk globule stage; in August they were either in the secondary or tertiary yolk globule stage. There was no effect of rearing temperature on oocyte stage in the Lemhi River females sampled in August.

In Rapid River spring chinook salmon males, GSI increase from May to July and then remained relatively constant until spawning (Figure 20). The increase in GSI during this period was associated with the progressive increase in proportion of spermatids, and spermatozoa (Figures 20 and 21). By August, testicular lobules of all males sampled were filled with spermatozoa, with few cysts of less mature germ cell types. Males were spermiating in September when milt could be collected with gentle abdominal pressure.

Hormone levels

(a) Insulin-like growth factor I (IGF I)--In the Rapid River fish, plasma levels of IGF I were significantly higher in males than females from May to August (Figure 22). Levels in males declined from August to September when there were no gender differences in IGF I. During the course of sampling, levels of IGF I in Rapid River females did not change and were significantly lower than that of the Lemhi River females in May and August. In August, plasma IGF I levels in Lemhi River females reared on chilled water were significantly lower than those in the fish reared on ambient water, but at the time of spawning IGF I levels were similar in these two groups.

(b) Gonadotropins--In the Rapid River spring chinook salmon males and females, pituitary levels of FSH increased from May to August, and declined at the time of spawning in September (Figure 23a). Gender differences in pituitary FSH content were substantial in August when males had about 40% higher levels than females. In contrast, during May and June, females had significantly higher FSH content than males, but this difference was small. The pituitaries of Lemhi River females had less than half the FSH content of Rapid River females at all time points (Figure 23a). In August and at spawning in September, pituitary FSH content was significantly lower in the fish reared on ambient water compared to the fish reared on chilled water. Plasma FSH profiles differed in male and female Rapid River chinook salmon (Fig. 23b). In males, plasma FSH levels were low relatively unchanged until spawning when levels declined slightly. In contrast, plasma FSH levels in Rapid River females increased from May to June, declined in July, and then increased again in August. Levels at spawning were more variable and not significantly different from that observed in August. In May and August, plasma FSH levels in Lemhi River females were not significantly different than that of the Rapid River females. However in August, levels of FSH in the chilled Lemhi females were significantly higher than those of the ambient females of the same stock. At spawning, FSH levels in the Lemhi River females were significantly lower than those

of the Rapid River females and there was no effect of rearing temperature in the Lemhi fish.

Pituitary LH content increased slightly from May to July (Figure 24a) in Rapid River spring chinook salmon males and females. From July to spawning, pituitary LH content increased 6-10 fold in both sexes. In May, pituitary LH content did not differ among the groups of fish examined. However, in August and at spawning, pituitary LH content in the Lemhi River females was significantly lower than that of the Rapid River females, and there was no effect of rearing temperature on LH content in the Lemhi River females. In the Lemhi River females, pituitary LH content increased from May to August, but no change was observed from August to spawning as was observed in the Rapid River fish. Plasma levels of LH in all groups were near the detection limit of the assay from May to August, and then increased at spawning (Figure 24b). However, the increase in plasma LH at spawning was greater in Rapid River females than in Rapid River males or Lemhi River females.

(c) Sex Steroids--Plasma levels of estradiol-17 β (E2) in Rapid River females were relatively constant from May to August (20-30 ng/ml), and declined to less than 1 ng/ml at spawning (Figure 25a). Levels of E2 in the Lemhi River females in May and at spawning were similar to that of the Rapid River females. However, in August, plasma levels of E2 in the Lemhi River females were extraordinarily high; almost three times that of the Rapid River females. Rearing temperature had no effect on plasma E2 levels in the Lemhi River females. In the Rapid River males, plasma 11-KT increased slightly from May to June, declined in July and then increased to peak levels in August and declined at spawning (Figure 25b).

Testosterone levels in Rapid River females showed a typical pattern of increasing to peak levels in late stages of secondary oocyte growth in August, and declining at spawning to levels about 50% of those in August (Figure 26a). In contrast, plasma levels of testosterone in Lemhi River females peaked at spawning in September, and were nearly 2-3 fold higher than that of the Rapid River females at this stage (Figure 26a). Mean levels of testosterone in male Rapid River Chinook salmon fluctuated between 25-50 ng/ml, and two peaks were observed in June and August.

A similar pattern of changes in plasma levels of 17,20 β -P was observed in Rapid River (both sexes) and Lemhi River chinook salmon over time (Figure 27). In Rapid River males and females, plasma 17,20 β -P remained low (0.5-2.5 ng/ml) from May to July, and increased significantly from July to August, and again at spawning. Levels increased 100-fold by the time of spawning in September. The magnitude of the increase in plasma 17,20 β -P in Lemhi River females was the same as that found in the Rapid River females, but the timing of the increase was delayed. Levels of 17,20 β -P in Lemhi River females in August were similar to that of all fish in May, but nearly 6-fold lower than that of the Rapid River females in August.

Spawning date and egg quality

Rapid River females spawned from mid-August through early September, while captive-reared Lemhi River females (NMFS reared fish) spawned from the second week of September through early October (Figures 28 and 29). Half of the Rapid River females were spawned by the last week of August, while the mid-point of spawning for the Lemhi River fish (NMFS-reared) was approximately 3 weeks later. No effect of temperature on spawn time in the captive Lemhi River females was observed (Figures 28-30). However, when the spawning times of Lemhi River fish reared in seawater at the MRS from May 1999 to June 2001 were compared with those of adults reared throughout their lives in fresh water at the IDFG Eagle Hatchery, a significant effect of rearing environment prior to June 2001 was found with the Eagle-reared fish spawning approximately 10 days later than the MRS-reared fish (Figure 30). In the Lemhi females, no effects of rearing environment on egg quality (survival of embryos to the eyed-stage) were detected (Figure 30), although Lemhi River females had significantly smaller eggs and higher relative fecundity than Rapid River females (Figure 31). Total fecundity of Rapid River females was four-fold higher than that of the Lemhi River females (Figure 32).

Data Management Activities

Data are collected manually by the research team onto preformatted data sheets or directly into PCs. Data are entered and summarized on personal computers operated by researchers using Microsoft Excel, Statview 512+, Cricket Graph 1.3.2, and Prism. Data on histology of tissues is recorded using a digital camera and stored as either TIFF or JPG files on a Power MacIntosh G4. Backup copies of data are saved on an external Lacie hard-drive and on CD-ROMs. All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report under Materials and Methods. Data analyses are reported in the Results section of the annual report.

Discussion and Conclusions

Size and composition

Overall, the patterns of changes in protein and lipid content in captive Lemhi River females were similar to the returning Rapid River hatchery females, but the timing was slightly delayed in the captive fish. The patterns of energy mobilization associated with reproductive maturation were similar to that reported for other Pacific salmon species (Idler and Bitners, 1958, 1959, 1960; Hardy et al. 1984; Hendry et al. 2000). The major differences between the captive and returning hatchery fish were that the Rapid River females were larger and leaner than captive Lemhi River females. The average fat and protein content of the soma and viscera of Rapid River spring chinook salmon declined over time as the fish matured reaching lowest levels at spawning. A similar pattern was

observed in captive Lemhi River females. However, prior to spawning captive Lemhi River females had 2-4 times higher visceral fat than the Rapid River females at the same time, yet levels at spawning were similar between the two groups. Maintaining the Lemhi River females in chilled fresh water slightly reduced the amount of lipid mobilized from both soma and viscera. Mean ovarian fat content declined while protein content increased as both Lemhi River and Rapid River females matured. However, in males, mean testicular fat and protein content increased as the fish matured.

No major differences in protein content of soma, viscera, or gonads were observed among the groups of females sampled on a given date except in May when levels of protein in the ovaries and viscera of Lemhi River females were substantially lower than the Rapid River fish. The ovarian protein content in the Lemhi River females may have been low in May because vitellogenesis was less advanced than the Rapid River females. In all groups of fish, the greatest relative changes in gonadal protein were associated with the greatest change in GSI. For males, this occurred from May to July, while in females this occurred from July to August.

Morphometrics

In the adults returning to the Rapid River Hatchery, an interesting increase in fin size was seen in males from August to September. Assuming this is not a sample artifact, large pelvic fins may be associated with male courtship behavior (e.g., signaling “size” to the female) or with milt release. Body form of sexually maturing male and female Rapid River chinook salmon changed over time. As described by a PC analysis, head morphology and trunk proportions changed gradually from May to August for both sexes. In September, male chinook salmon exhibited a drastic change in head and trunk proportions. Bivariately, this change involved a significantly larger jaw/lower head in males in September. This ontogenetic pattern was seen less dramatically in a relative warp analysis of body outlines. The timing of these changes coincides with other dramatic physiological changes at this period including increases in plasma sex steroids (11-KT and 17, 20 β -P) and plasma LH. It is assumed that these morphological changes are associated with enhanced performance, success, and survival during mating which commences in August.

Samples from captive broodstock (Lemhi River and Catherine Creek stocks) were not collected in the same temporal sequence, and were essentially from females only collected in May and August (Lemhi only). The two samples of female captive broodstock were different from the returning hatchery Rapid River female collection in May, exhibiting PC2 values of returning hatchery females in June-August. Previous studies have shown that cultured salmon have different body proportions and shapes than fish reared in the wild (Fleming et al. 1994; Hard et al. 2000). Clearly, captively-reared spring chinook females differed in the timing of specific morphological changes associated with sexual maturation.

Gonad development

At the first sampling in May, the oocytes of all Rapid River and Lemhi River females were in various stages of secondary oocyte growth. However, the oocytes of Rapid River females were more advanced and more uniform in the ovarian development than the Lemhi River females. In May, the oocytes of all of the Rapid River females were in the secondary yolk globule stage, while many of the Lemhi River females were still in the primary yolk globule stage. This difference in relative stage continued through the August sampling. By early August, the oocytes of all of the Rapid River females had advanced to the tertiary yolk globule stage; whereas, only some of the Lemhi River females had advanced to this stage and the degree of advancement was not dependent on rearing temperature.

The data on ovarian development in the Lemhi River females are somewhat difficult to interpret because of the high degree of ovarian atresia that was observed in a large number of the Lemhi River females, primarily in the left ovary. It is not known to what degree the atresia influenced the rate of ovarian development of the healthy oocytes. However, if only healthy oocytes are considered, it is apparent that the oocytes of captive Lemhi River females were less advanced than the Rapid River females from the outset of the experiment, and alterations in rearing environment were not adequate to change the rate of oocyte growth sufficiently to catch up to the Rapid River females, or wild anadromous Lemhi River females.

Gastric distention

The atresia and hemorrhaging observed in the ovaries of captive Lemhi River females and Catherine Creek females reared in seawater at the MRS were associated with gross distention of the stomach, which was filled with copious quantities of oil and watery fluid. The gastric distention and ovarian atresia was observed in 7 out of 8 Lemhi River females sampled in May. By August, after three months of fasting, the gastric distention was not apparent, however ovarian abnormalities remained in half of the fish. In some cases the left ovary was completely regressed while in others the left ovary was twisted and formed a U-shape around the stomach. In May, this condition was unique to fish reared in seawater, as Catherine Creek spring chinook salmon of the same year class reared in fresh water at the Bonneville Hatchery did not show this disorder while those reared in seawater did. The gastric distention appeared to be a chronic condition that caused compression atrophy of the ovary, particularly as the ovary enlarged during vitellogenesis. In many cases the stomach was dilated and flaccid, without rugal folds. In extreme cases, the left ovary was almost completely regressed while in other individuals the ovary twisted and grew around the stomach, and severe hemorrhaging occurred at the point of constriction in the ovary. Adhesions were frequently observed between the ovary and digestive track. The ovarian regression was largely confined to the left ovary probably because of the anatomical arrangement of the internal organs where the liver is primarily on the ventral and right side, and the stomach is positioned slightly to the left side. Thus, the expansion of the stomach would be more likely to impact the left ovary. The gastric distention was often not apparent externally. This is in

contrast to cases reported in farmed New Zealand chinook salmon (Lumsden et al. 2002), sea-farmed rainbow trout (Staurnes et al. 1990), and observations in other captive broodstock, for example, in Lake Wenatchee sockeye salmon (C. McAuley, NMFS, personal communication). The abdominal musculature of the spring chinook salmon captive broodstock examined in the present study was substantially thicker than that of the Lake Wenatchee sockeye salmon broodstock, and may have acted as a 'girdle' to restrict organs spatially in the peritoneal cavity.

At present, the underlying cause of gastric distention in the captive Lemhi River and Catherine Creek chinook salmon reared in saltwater are not known. Similar gastric distention disorders have been documented in farmed chinook salmon reared in seawater in net pens in New Zealand (Lumsden et al. 2002) or British Columbia (Hicks 1989), and Atlantic salmon in Norway (Staurnes et al. 1990). Gastric dilation could be induced in juvenile rainbow trout fed diets containing histamine, polyamines, or stale fish meal (Watanabe et al. 1987, Fairgrieve et al. 1994). However, none of the aforementioned studies examined the impacts to gonadal development in broodstock. Several reports of this syndrome in salmon have indicated that it can appear rapidly after saltwater transfer, suggesting an association with some aspect of osmoregulation. Indeed, many investigators and hatchery managers have concluded that the gastric distention is a result of inappropriate timing of saltwater transfer of smolts, or inappropriate smoltification. In support of this are the results of a study by Rorvik et al. (2000), who demonstrated that gastric distention occurred after inducing osmoregulatory stress by reducing rearing temperature in rainbow trout in seawater. However, Lumsden et al. (2002) noted that gastric distention occurred to a lesser degree in chinook salmon reared on the same diets in fresh water and concluded that osmoregulatory difficulties were probably a consequence of the disorder and not a cause.

Several causative agents in fish feed have been identified including presence of biogenic amines (e.g. histamine, gizzerosine) or polyamines (cadavarine) that are present in stale fish meal, or created upon heating fish meal during processing of diets (Lumsden et al. 2002, Fairgrieve et al. 1994). Lumsden et al. (2002) and Hicks (1989) also mention that there are species and stock differences in the sensitivity to dietary components that induce this disorder; however, no reports are available that document this. Clearly not all of the fish examined in the present study displayed this disorder (Appendix I); therefore, the response to the causative agent is not uniform in the spring chinook salmon captive broodstock. At the MRS, gastric distention has been recently observed in coho salmon (University of Washington, stock) and spring chinook salmon (Carson, Lemhi River, and Catherine Creek stocks), all of which were fed the same diets and reared in the same water source. The Carson spring chinook salmon females also displayed atresia in the ovary that was associated with gastric distention (Swanson et al. 2003). The UW coho salmon were not reared to full maturity so the impacts to the ovary were not assessed. Not all fish displayed the disorder suggesting individual differences in sensitivity to the causative agent, or differences in osmoregulatory function perhaps due to developmental differences in the fish at that time of transfer to seawater.

Despite the high incidence of gastric distention disorder in seawater, the captive Lemhi River broodstock matured and in some cases produced eggs with high fertility and embryos with high survival. Given the observed ovarian atresia in the broodstock reared in seawater, one would expect large fecundity differences between broodstock reared in seawater versus fresh water. However, fecundity and egg quality of Catherine Creek spring chinook salmon broodstock reared in seawater at the MRS did not differ substantially from those reared in fresh water at the Bonneville Hatchery. In fact, the fish reared in seawater had smaller eggs and higher relative fecundity (T. Hoffnagle, ODFW, personal communication), which is contrary to what would be expected if massive atresia of oocytes was occurring in the seawater-reared fish, and affecting egg fertility and number.

Sex steroids

The changes in plasma sex steroids and gonadotropins during maturation of the spring chinook salmon females observed in this study are similar to that reported for Willamette River spring chinook salmon females (Slater et al. 1994), as well as many other salmonid fish (Sower and Schreck 1982, Fitzpatrick et al. 1986, Truscott et al. 1986, Sumpter and Scott 1989). The classic shift from E to 17,20 β -P production that occurs at the end of vitellogenesis and during the onset of final oocyte maturation was observed in both Rapid River and captive Lemhi River females. However, it is noteworthy that the Lemhi River females had extraordinarily high levels of E at the August sampling, compared with the Rapid River females and that of previous studies (Slater et al. 1994). This may be in part due to the fact that the oocytes of the Lemhi River females were at earlier stages of secondary oocyte growth than the Rapid River females at this time. However, the levels of E were much greater than would be expected for this stage. It is possible that the high E levels occurred as a result of atresia observed in the ovary, as the normal feedback relationships between the ovary and pituitary gland would be disturbed. If this were true, one would expect abnormal levels of other gonadal steroids, which was not the case for the Lemhi River females. While T levels at spawning were higher in the Lemhi River than the Rapid River females, they are well within the range observed in other stocks of spring chinook salmon females (e.g. Slater et al. 1994). The patterns of changes in plasma 17,20 β -P in the Rapid River and captive Lemhi River females were similar to that reported in other salmonids; levels are low during secondary oocyte growth and increase during final oocyte maturation and ovulation. However, the levels of 17,20 β -P at spawning were lower in the captive Lemhi River females than the Rapid River females, which may be due to the lower ovarian mass relative to body weight in the Lemhi River females.

In the Rapid River males, androgen (11-KT and T) levels increased as spermatogenesis progressed, while 17,20 β -P levels increased with increase in the proportion of spermatids and spermatozoa in the testis, and spermiation. This is similar to what has been reported for other salmonids (Schulz 1984, Planas and Swanson 1995, Prat et al. 1996).

Gonadotropins

Previous studies in salmon have shown that gonadal growth and maturation are regulated primarily by pituitary gonadotropins, FSH and LH. FSH regulates early stages of gametogenesis and is released in high levels during secondary oocyte growth and spermatogenesis. On the other hand, LH levels remain low or non-detectable during gonadal growth and increase during final oocyte maturation, ovulation and spermiation (Swanson 1991). Therefore, levels of gonadotropins in both the pituitary and plasma were measured to determine if there were abnormalities in the patterns of gonadotropin release or production in the captive broodstock compared with the Rapid River fish.

Plasma levels of gonadotropins in the Rapid River females were similar to that reported previously in Willamette River spring chinook salmon (Slater et al. 1994), except that FSH levels were substantially lower and more variable. The plasma FSH levels in the Lemhi River females were also lower than that reported for other salmonids at comparable stages of oogenesis (Swanson 1991, Oppen-Bernston et al. 1994, Prat et al. 1996, Gomez et al. 1999, Davies et al. 1999). In addition, the increase in LH, which induces ovulation, was not as great in the Lemhi River females compared with the Rapid River females. This may be due to inadequate (environmental or pheromonal) cues to induce a large pre-ovulatory surge in LH. Analysis of pituitary LH and FSH content revealed that both gonadotropins were lower in the Lemhi River females compared with that of the Rapid River females. This difference could not be attributed solely to body and pituitary size differences in the fish. The Lemhi females were half the body size of Rapid River females, yet pituitary gonadotropin content was 3-fold lower at spawning. Because the ovary produces steroids and peptides that feedback on the pituitary to regulate gonadotropin biosynthesis (Yaron et al. 2003), the Lemhi females may have reduced gonadotropins because of the reduced ovarian mass relative to body size. It is also possible that stress of the captive environment may have altered gonadotropin production in the Lemhi River broodstock. In brown trout, stress has been shown to reduce gonadotropin levels (Pickering et al. 1987).

Insulin-like growth factor I

Previous studies have shown that plasma levels of IGF I are related to growth rate (Beckman et al. 2001) except in maturing fish. Increases in plasma IGF I associated with the progression of gonadal maturation have been reported for spring chinook salmon males (Shearer and Swanson 2000). In the present study, plasma IGF I levels were two to four-fold higher in Rapid River males than females. In males, plasma IGF I levels remained elevated until spawning when levels declined to that of females. The low IGF I levels in females were expected, as all fish were fasting and prolonged fasting has been shown to reduce IGF I levels (Pierce et al. 2001). However, in males, IGF I levels appeared to be related to maturation rather than feeding status. This suggests that IGF I may play an important role in regulating spermatogenesis in salmon as well as body growth.

Spawning time

The mean spawning time of captive Lemhi River females was 3-4 weeks after that of the Rapid River fish. Although Lemhi River females reared on chilled water tended to spawn earlier, this difference was not significant. This result was unexpected, because in Atlantic salmon reduced water temperatures at the time of transfer of adults to fresh water advanced spawn time of females by 2 weeks (Taranger et al. 1999). However, a decline in water temperature may be a more important cue for spawning in Atlantic salmon in Northern latitudes that spawn in October and November than Snake River spring chinook salmon that spawn in August and September. The delay of spawning of the Lemhi River females relative to the Rapid River females is not surprising given that oocyte development in the Lemhi River females was behind that of the Rapid River females at the earliest sampling in May. This suggests that rearing environment or rearing practices prior to transfer of adults to fresh water are not promoting appropriate timing or rate of ovarian growth. In support of this conclusion is the observation that Lemhi River females reared at the Eagle Hatchery spawned later than those reared at the MRS, even though the rearing environments from June to September (spawning) were identical.

The underlying causes of the delayed oocyte growth are not known, but could be due to rearing temperature and/or growth regimes. Recent studies in coho salmon (Campbell et al. 2003) have shown that the degree of advancement of oocytes in early stages of secondary growth was related to body size in May, 6-7 months prior to spawning. Therefore, present investigations are focused on how growth and/or rearing temperature during the seawater phase of the life cycle influences ovarian growth and spawning time in both coho and spring chinook salmon.

Captive Lemhi River females produced smaller and fewer eggs than the Rapid River females; however, relative fecundity was higher than that of the Rapid River fish. These differences are not surprising because growth affects both egg size and egg number, and the Lemhi River females were half the size of the Rapid River females. The fecundities of the Lemhi River females could have also been affected by the degree of ovarian atresia that was observed, particularly in some cases where the left ovary was almost completely regressed.

References

- Beckman, B.R., K.D. Shearer, K.A. Cooper, and W.W. Dickhoff. 2001. Relationship of insulin-like growth factor-I and insulin to size and adiposity of underyearling chinook salmon. *Comp. Biochem. Physiol.* 129A:585-593.
- Beeman, J.W., D.W. Rondorf, and M.E. Tilson. 1994. Assessing smoltification of juvenile spring chinook salmon (*Oncorhynchus tshawytscha*) using changes in body morphology. *Can. J. Fish. Aquat. Sci.* 51: 836-844.
- Billard, R., and B. Breton. 1977. Sensibilité à la température des différentes étapes de la reproduction chez la truite arc-en-ciel. *Cah. Lab. Hydrobiol. Montereau* 5:5-24.
- Bookstein, F.L. 1991. *Morphometric tools for landmark data: geometry and biology.* Cambridge University Press, New York. 435 pp.
- Campbell, B., B.R. Beckman, W.T. Fairgrieve, J.T. Dickey, and P. Swanson. 2003. Effects of growth on maturation timing, fecundity, and egg size in coho salmon. Final Report. In B.A. Berejikian and C.E. Nash (editors), FY 2002 Annual report to Bonneville Power Administration, Project Number 9350056.
- Cadrin, S.X. 2000. Advances in morphometric identification of fishery stocks. *Rev. Fish Bio. Fisheries* 10: 91-112.
- Cuisset, B., P. Pradelles, and D.E. Kime. 1994. Enzyme immunoassay for 11-ketotestosterone using acetylcholinesterase as label: application to measurement of 11-ketotestosterone in Siberian sturgeon. *Comp. Biochem. Physiol.* 108C: 229-241.
- Davies, B., N. Bromage, and P. Swanson. 1999. The brain-pituitary-gonadal axis of female rainbow trout (*Oncorhynchus mykiss*): Effects of photoperiod. *Gen. Comp. Endocrinol.* 115:155-166.
- Fairgrieve, W.T., M.S. Meyers, R.W. Hardy, and F.M. Dong. 1994. Gastric abnormalities in rainbow trout (*Oncorhynchus mykiss*) fed amine-supplemented diets or gizzard-erosion positive fish meal. *Aquaculture* 127:219-232.
- Fitzpatrick, M.S., G. Van Der Kraak, and C.B. Schreck. 1986. Plasma profiles of sex steroids and gonadotropin in coho salmon (*Oncorhynchus kisutch*) during final maturation. *Gen. Comp. Endocrinol.* 62:437-451.
- Fleming, I.A., B. Jonsson, and M.R. Gross. 1994. Phenotypic divergence of sea-ranched, farmed, and wild salmon. *Can. J. Fish. Aquat. Sci.* 51:2808-2824.
- Gomez J. M., C. Weil, M. Ollitrault, P-Y. Le Bail, B. Breton, and F. Le Gac. 1999. Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 113:413-428.
- Hard, J.J., G.A. Winans, and J.C. Richardson. 1999. Phenotypic and genetic architecture of juvenile morphometry in chinook salmon. *J. Hered.* 90:597-606.
- Hard, J.J., B.A. Berejikian, E.P. Tezak, S. L. Schroder, C.M. Knudsen, and L.T. Parker. 2000. Evidence for morphometric differentiation of wild and captive-reared adult coho salmon: a geometric analysis. *Environ. Biol. Fish.* 58:61-73.
- Hardy, R.W., K.D. Shearer, and I.B. King. 1984. Proximate and elemental composition of developing eggs and maternal soma of pen-reared coho salmon (*Oncorhynchus*

- kisutch*) fed production and trace element fortified diets. *Aquaculture* 43:147-165.
- Hendry, A.P., A.H. Dittman, and R.W. Hardy. 2000. Proximate composition, reproductive development, and a test for trade-offs in captive sockeye salmon. *Trans. Am. Fish. Soc.* 129:1082-1095.
- Hicks, B. 1989. *British Columbia Salmonid Disease Handbook*. British Columbia Ministry of Agriculture and Fisheries, Vancouver, Canada. p.40
- Idler, D. R., and I. Bitners. 1958. Biochemical studies on sockeye salmon during spawning migration. II. Cholesterol, fat, protein, and water in the body of the standard fish. *Can. J. Biochem. and Physiol.* 36:793-798.
- Idler, D. R., and I. Bitners. 1959. Biochemical studies on sockeye salmon during spawning migration. V. Cholesterol, fat, protein, and water in the body of the standard fish. *J. Fish. Res. Bd. Canada* 16:235-241.
- Idler, D.R., and I. Bitners. 1960. Biochemical studies on sockeye salmon during spawning migration. IX. Fat, protein, and water in major internal organs and cholesterol in liver and gonads of the standard fish. *J. Fish. Res. Bd. Canada* 16:559-560.
- Kinnison, M., M. Unwin, N. Boustead, and T. Quinn. 1998. Population-specific variation in body dimensions of adult chinook salmon (*Oncorhynchus tshawytscha*) from New Zealand and their source population, 90 years after introduction. *Can. J. Fish. Aquat. Sci.* 55: 554-563.
- Loir, M. 1999. Spermatogonia of rainbow trout: I. Morphological characterization, mitotic activity and survival in primary cultures of testicular cells. *Mol. Reprod. Dev.* 53:422-433.
- Lumsden, J.S., P. Clark, S. Hawthorn, M. Minamikawa, S.G. Fenwick, M. Haycock, and B. Wybourne. 2002. Gastric dilation and air sacculitis in farmed chinook salmon, *Oncorhynchus tshawytscha* (Walbaum). *J. Fish Diseases* 25:155-163.
- Nagahama, Y. 1983. The functional morphology of teleost gonads. In W.S. Hoar, D.J. Randall, and E.M. Donaldson (editors), *Fish Physiology* (Volume IXA, pp. 223-275. Academic Press, New York.
- Oppen-Berntsen, D.O., S.O. Olsen, C.J. Rong, G.L. Tarranger, G.L., P. Swanson, and B.T. Walther. 1994. Plasma levels of eggshell ZR-proteins, estradiol-17 β , and gonadotropins during an annual reproductive cycle of Atlantic salmon (*Salmo salar*). *J. Exp. Zool.* 268:59-70.
- Pickering, A.D., T.G. Pottinger, J. Carragher, and J.P. Sumpter. 1987. The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout (*Salmo trutta* L.). *Gen. Comp. Endocrinol.* 68:249-259.
- Pierce A.L., B.R. Beckman, K.D. Shearer, D.A. Larsen, and W.W. Dickhoff. 2001. Effects of ration on somatotrophic hormones and growth in coho salmon. *Comp. Biochem. Physiol.* 128B:255-264.
- Planas, J.V., and P. Swanson. 1995. Maturation-associated changes in the response of the salmon testis to the steroidogenic actions of gonadotropins (GTH I and GTH II) in vitro. *Biol. Reprod.* 52:697-704.
- Prat F., J.P. Sumpter, and C.R. Tyler. 1996. Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations

- throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biol. Reprod. 54:1375-1382.
- Rodriguez, L., I. Begtashi, S. Zanuy, and M. Carillo. 2000. Development and validation of an enzyme immunoassay for testosterone: Effects of photoperiod on plasma testosterone levels and gonadal development in male sea bass (*Dicentrarchus labrax*, L.) at puberty. Fish. Physiol. Biochem. 23:141-150.
- Rohlf, F.J. 1998. *TPSRELV*: Relative warps analysis. Version 1.14. Dept. of Ecology and Evolution, State University of New York, Stony Brook.
- Rohlf, F.J. 1998. *tpsDig*: Program for digitizing images. Version 1.18. Dept. of Ecology and Evolution, State University of New York, Stony Brook.
- Rorvik K.-A., P.O. Skjervold, S.O. Fjaera, and S.H. Stein. 2000. Distended, water-filled stomach in seawater farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), provoked experimentally by osmoregulatory stress. J. Fish Diseases 23:15-18.
- Schulz R.W. 1984. Serum levels of 11-oxotestosterone in male and 17 β -estradiol in female rainbow trout (*Salmo gairdneri*) during the first reproductive cycle. Gen. Comp. Endocrinol. 56:111-120.
- Scott, A.P., E.L. Sheldrick, and P.F. Flint. 1982. Measurement of 17 α , 20 α -dihydroxy-4-pregnen-3-one in plasma of trout (*Salmo gairdneri*): seasonal change and response to salmon pituitary extract. Gen. Comp. Endocrinol. 46: 444-451.
- Shimizu, M., P. Swanson, and W.W. Dickhoff. 2000. Comparison of extraction methods and assay validation for salmon insulin-like growth factor I using commercially available components. Gen. Comp. Endocrinol. 119:26-36.
- Shearer K.D., and P. Swanson. 2000. The effect of whole body lipid on early sexual maturation of 1+ age male chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture 190:343-367.
- Slater, C.H., C.B. Schreck, and P. Swanson. 1994. Plasma profiles of sex steroids and gonadotropins in maturing female spring chinook salmon (*Oncorhynchus tshawytscha*). Comp. Biochem. Physiol. 109A: 167-175.
- Sower, S. A., and C. B. Schreck. 1982. Steroid and thyroid hormones during sexual maturation of coho salmon (*Oncorhynchus kistuch*). Gen. Comp. Endocrinol. 47:42-53.
- Staurnes, M., G. Andorsdottir, and A. Sundby. 1990. Distended, water-filled stomach in sea-farmed rainbow trout. Aquaculture 90:333-343.
- Sumpter, J.P., and A.P. Scott. 1989. Seasonal variations in plasma and pituitary levels of gonadotropins in males and females of the strains of rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 75:376-388.
- Swanson, P., M. Bernard, M. Nozaki, K. Suzuki, H. Kawauchi, and W.W. Dickhoff. 1989. Gonadotropins I and II in juvenile coho salmon. Fish Physiol. Biochem. 7: 169-176.
- Swanson, P. 1991. Salmon gonadotropins: Reconciling old and new ideas. In, A.P. Scott and J.P. Sumpter (editors), Reproductive Physiology of Fish, pp.2-7. Univ. of East Anglia, Norwich, England.
- Swanson, P., B. Campbell, J. Dickey, N. Hodges, R. Endicott, and B. Berejikian. 2003. Effects of water temperature during seawater rearing on timing of spawning and egg quality in spring chinook salmon. Progress Report. In B.A. Berejikian and

- C.E. Nash (editors), FY2002 Annual Report to Bonneville Power Administration Project 9350056.
- Taranger, G.L., S.O. Stefansson, F. Oppedal, E. Andersson, T. Hansen, and B. Norberg. 1999. Photoperiod and temperature affects gonadal development and spawning time in Atlantic salmon (*Salmo salar* L.). In Proc. 6th International Symposium on the Reproductive Physiology of Fish, July 4-9, 1999, p.345. University of Bergen, Norway.
- Truscott, B., D.R. Idler, Y.P. So, and J.M. Walsh. 1986. Maturation steroids and gonadotropin in upstream migratory sockeye salmon. Gen. Comp. Endocrinol. 62:99-110.
- Watanabe, T., T. Takeuchi, S. Satoh, K. Toyama, and M. Okuzumi. 1987. Effect of histidine or histamine on growth and development of stomach erosion in rainbow trout. Nippon Suisan Gakkaishi 53:1207-1214.
- Winans, G.A. 1984. Multivariate morphometric variation in Pacific salmon. I. A technical demonstration. Can. J. Fish. Aquat. Sci. 41(8):1150-1159.
- Winans, G.A., and R.S. Nishioka. 1987. Multivariate description of change in body shape of coho salmon (*Onchorynchus kisutch*) during smoltification. Aquaculture 66:235-245.
- Winans, G.A., S. Pollard, and D.R. Kuligowski. 2003. Two reproductive life history types of kokanee, *Onchorynchus nerka*, exhibit multivariate and protein genetic differentiation. Environ. Biol. Fish., in press:1-14.
- Yaron, Z., G. Gur, P. Melamed, H. Rosenfeld, A. Elizur and B. Levavi-Sivan. 2003. Regulation of fish gonadotropins. Int. Rev. Cytol. 225:131-185.

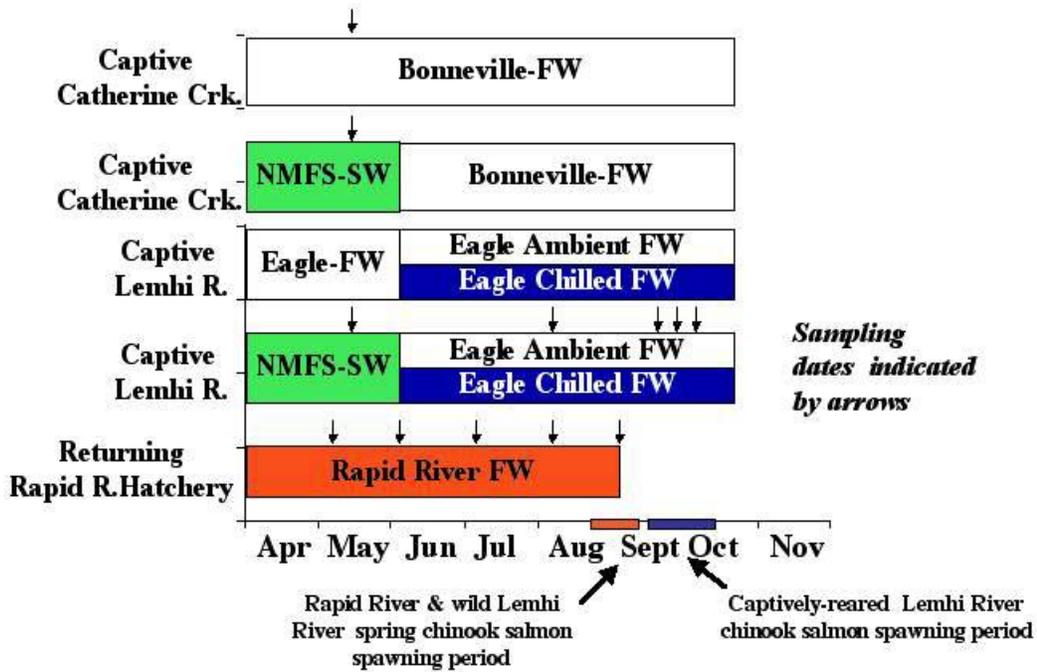


Figure 1. Rearing regimes and sampling times. FW, fresh water. SW, seawater.

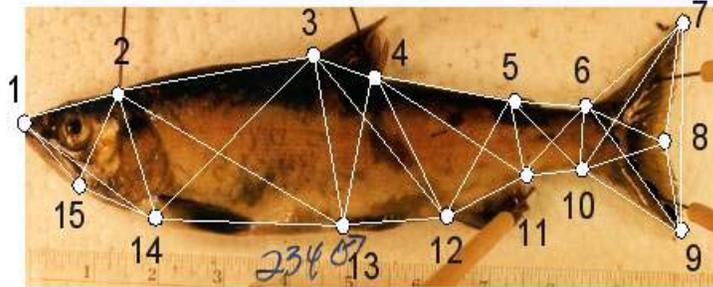


Figure 2. Location of 15 landmarks on the body outline. 35 inter-landmark distances are illustrated. Four landmarks used to calculate dorsal and anal fin heights, and pectoral and pelvic fin length are not shown.

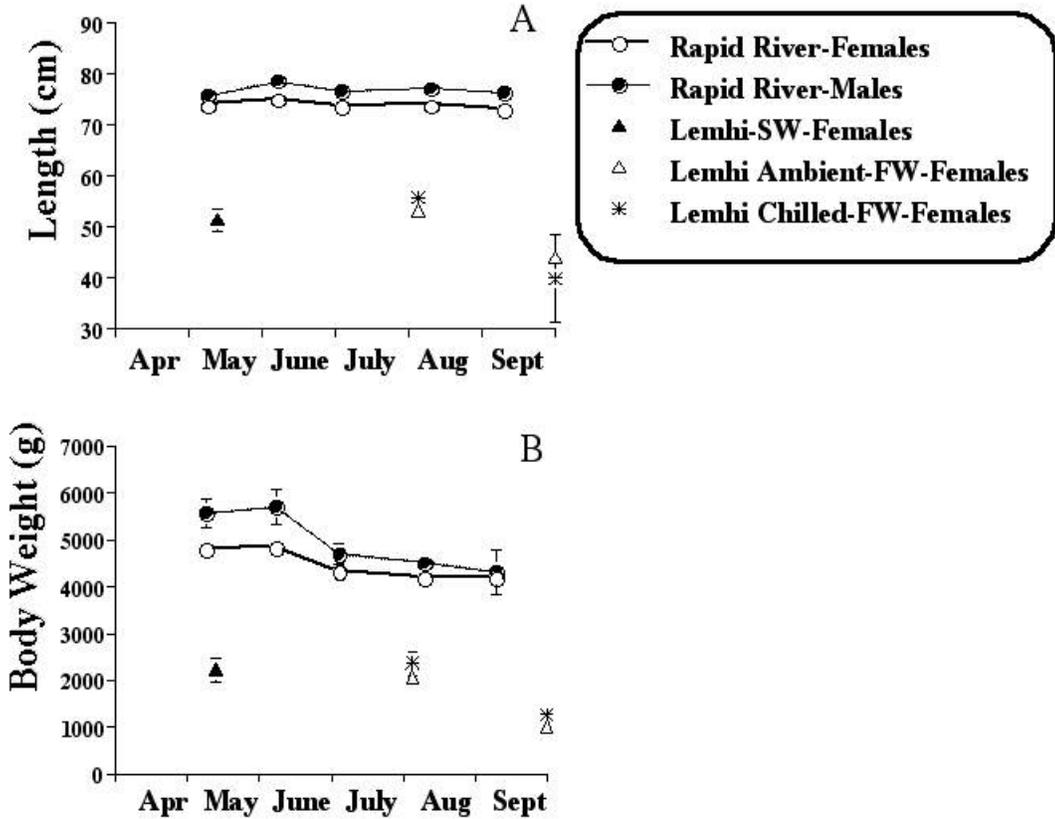


Figure 3. Body length (A) and weight (B) for male and female Rapid River, female Lemhi River spring chinook salmon sampled during the course of the study. Data are mean \pm SE of n=5-10 fish per sample date. Error bars smaller than the data point are not shown.

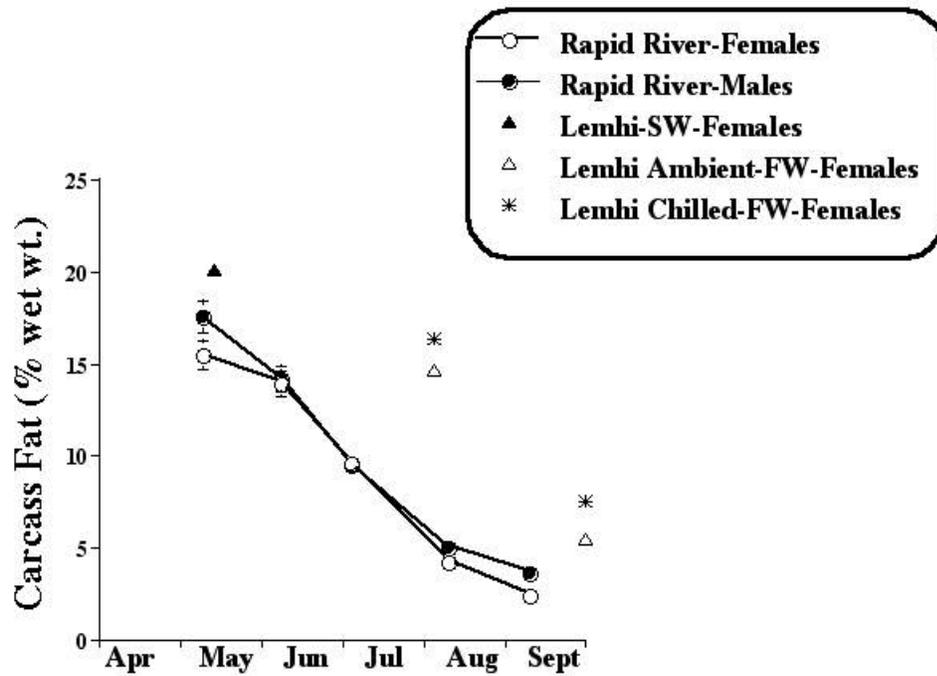


Figure 4. Carcass fat content for male and female Rapid River, female Lemhi River spring chinook salmon sampled during the course of the study. Data are mean \pm SE of n=5-10 fish per sample date. Error bars smaller than the data point are not shown.

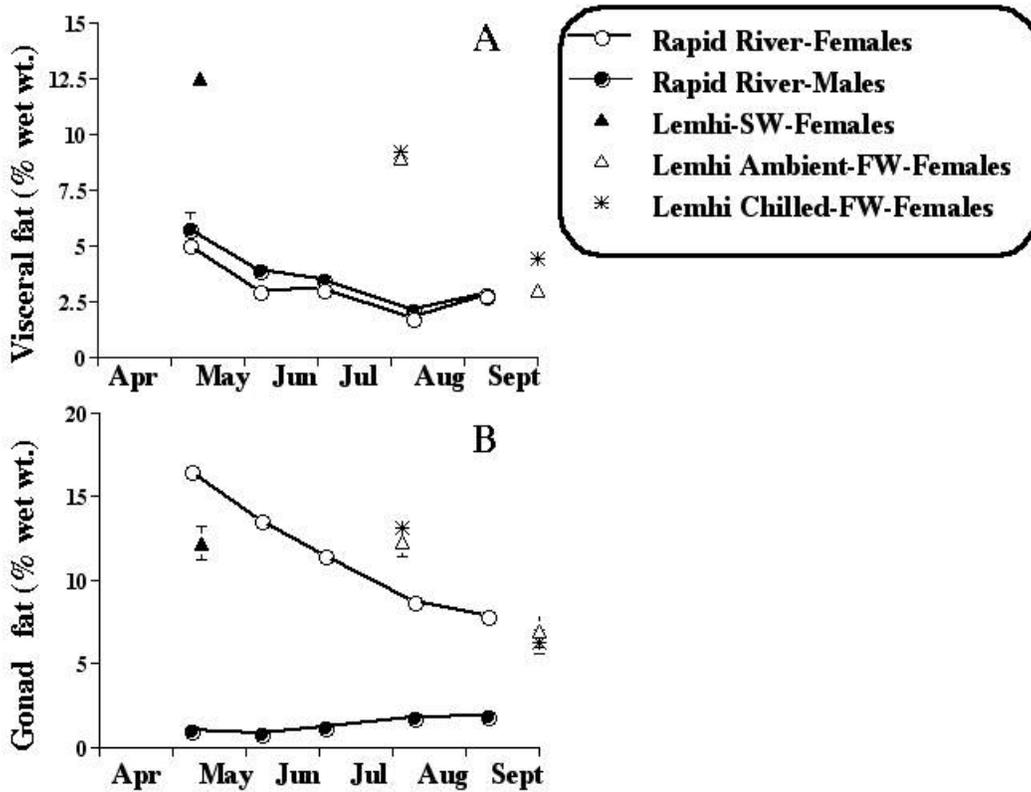


Figure 5. Visceral fat (A) and gonad fat (B) content for male and female Rapid River, female Lemhi River spring chinook salmon sampled during the course of the study. The samples collected in September from females are ovulated eggs. Data are mean \pm SE of n=5-10 fish per sample date. Error bars smaller than the data point are not shown.

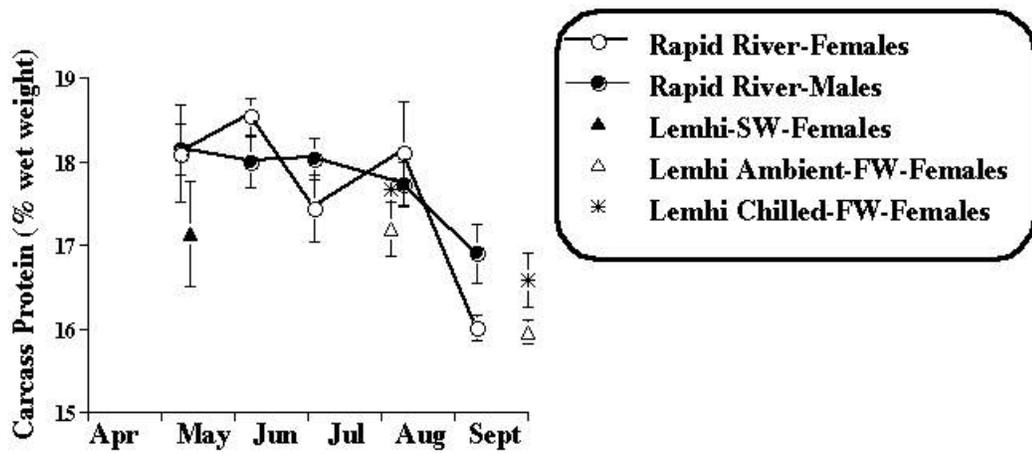


Figure 6. Carcass protein content for male and female Rapid River, female Lemhi River spring chinook salmon sampled during the course of the study. Data are mean \pm SE of n=5-10 fish per sample date. Error bars smaller than the data point are not shown.

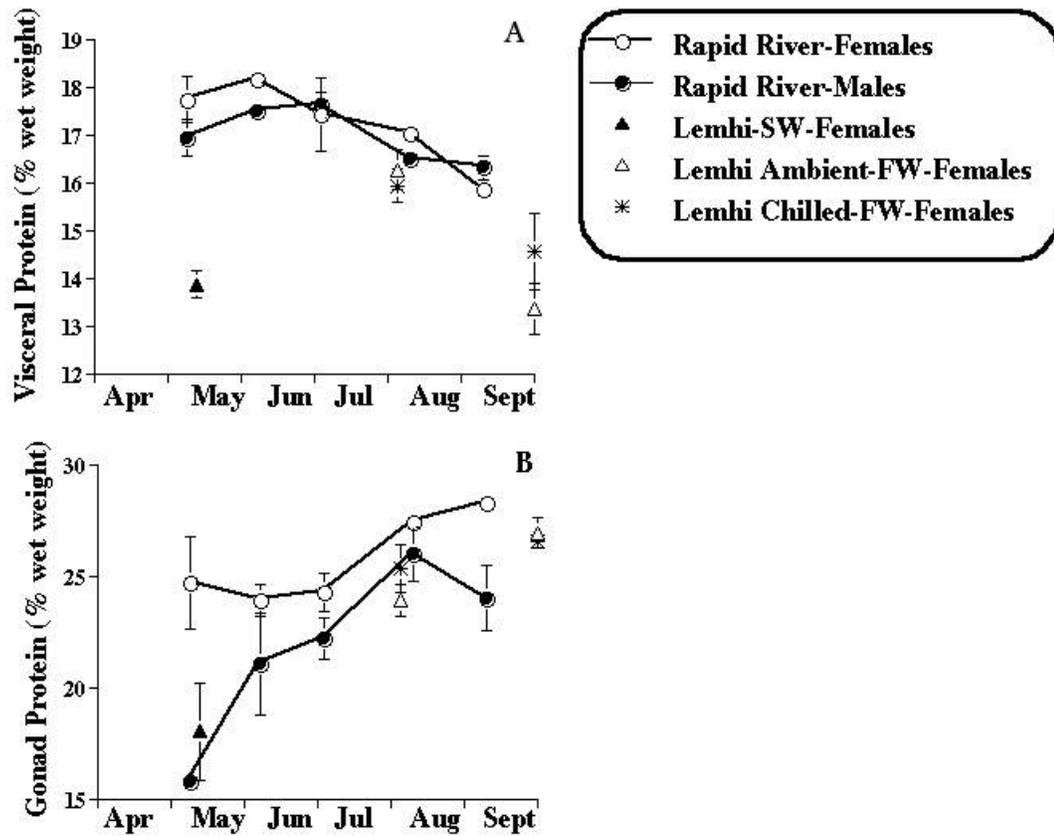


Figure 7. Visceral protein (A) and gonad protein (B) content for male and female Rapid River, female Lemhi River spring chinook salmon sampled during the course of the study. The samples collected in September from females are ovulated eggs. Data are mean \pm SE of n=5-10 fish per sample date. Error bars smaller than the data point are not shown.

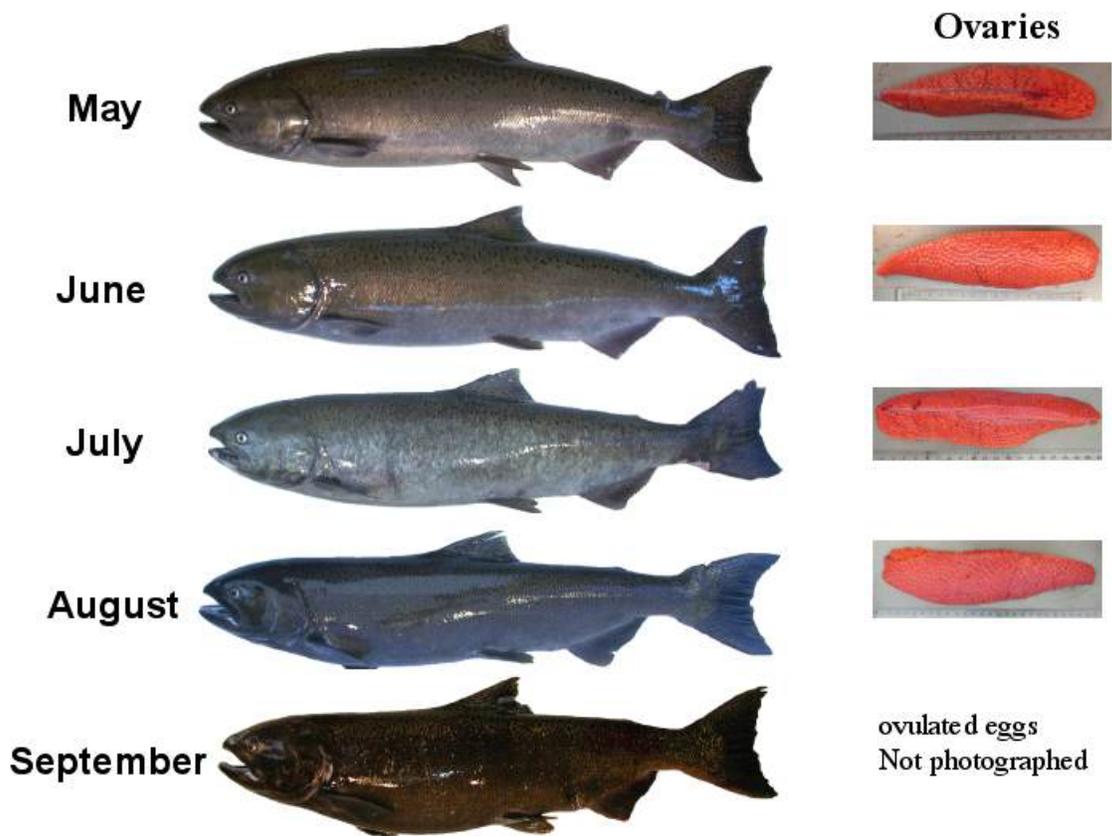


Figure 8. External body morphology and gross morphology of the gonads of Rapid River female spring chinook salmon collected monthly from May through September 2001 at the IDFG Rapid River Hatchery, Riggins, ID. Samples from 10 fish per month were collected but only representative photographs are shown.

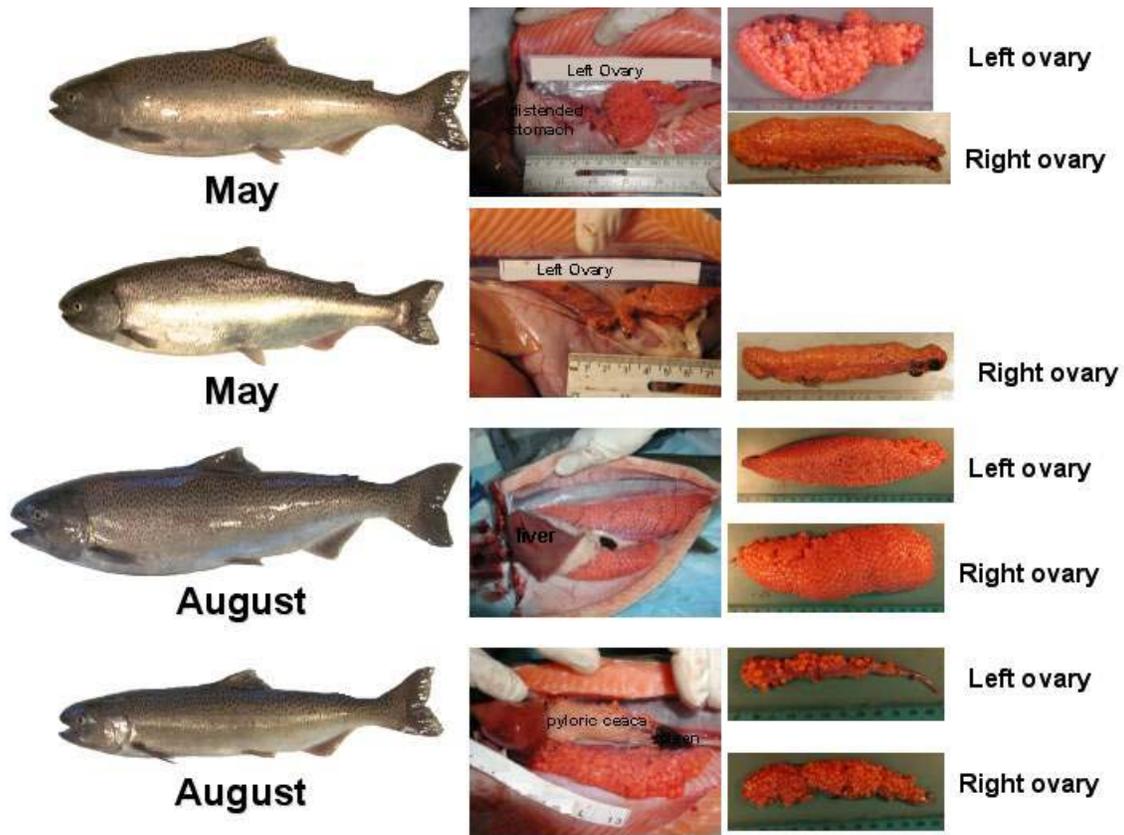


Figure 9. External body morphology and gross morphology of the gonads of female Lemhi River spring chinook salmon captive broodstock collected in May 2001 at Manchester Research Station, Manchester, WA and in August 2001 at the IDFG Eagle Fish Hatchery, Eagle ID. Samples from 8-9 fish per month were collected, but only representative photographs are shown in this figure. Data on ovarian morphology from and histology of all Lemhi River samples collected are shown in Appendix 1.

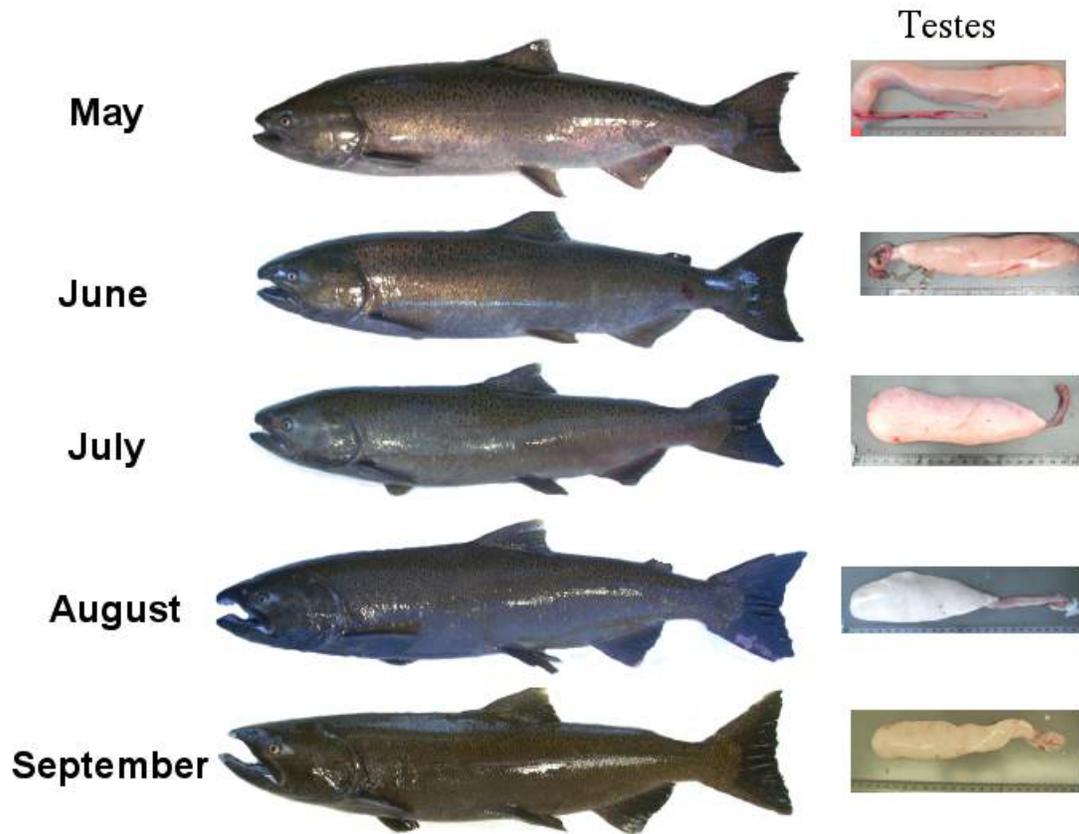


Figure 10. External body morphology and gross morphology of the gonads of Rapid River male spring chinook salmon collected monthly from May through September 2001 at the IDFG Rapid River Hatchery, Riggins, ID. Samples from 10 fish per month were collected but only representative photographs are shown.

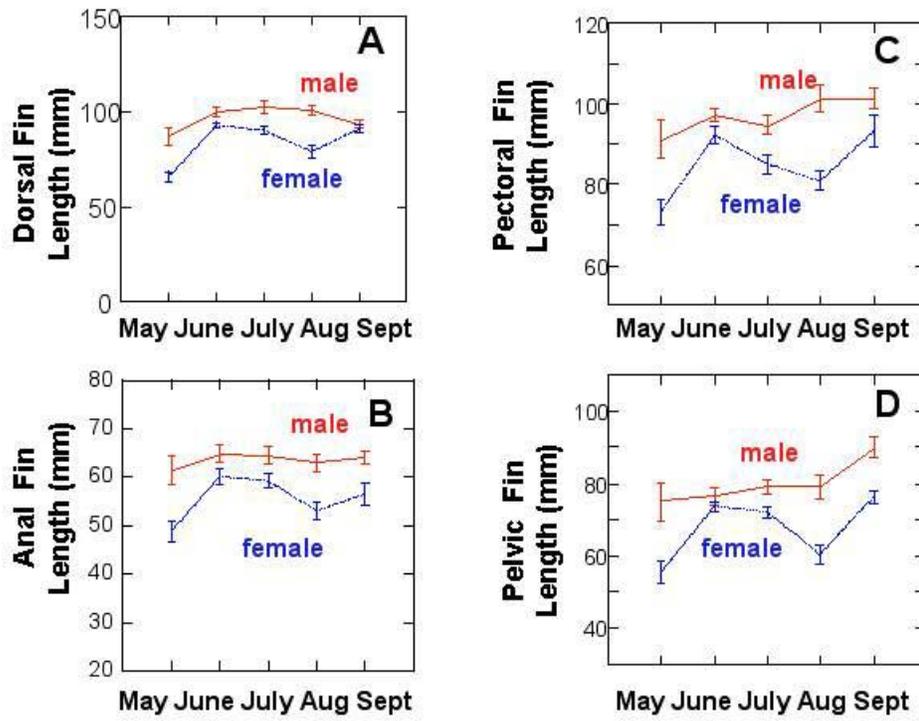


Figure 11. Mean fin length ($\pm 2SE$) for dorsal (A), anal (B), pectoral (C), anal, and pelvic (D) fins for Rapid River males and females (n=10/data point).

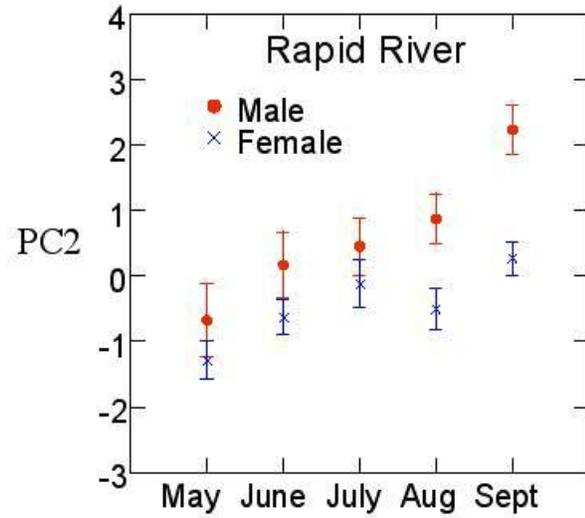


Figure 12. Distribution of principal component (PC) 2 scores (± 2 SE), $n=10$ /data point, for Rapid River spring chinook salmon ($n= 10$ /data point).

PC-2

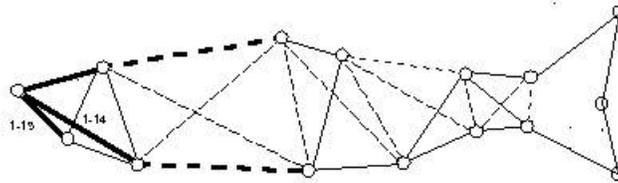


Figure 13. Important morphometric distances in principle component (PC) analyses of 26 distance characters for PC2. Weighting coefficients for these characters are + (bold solid lines) or – (bold dashed lines). Body outline is included for illustrative purposes.

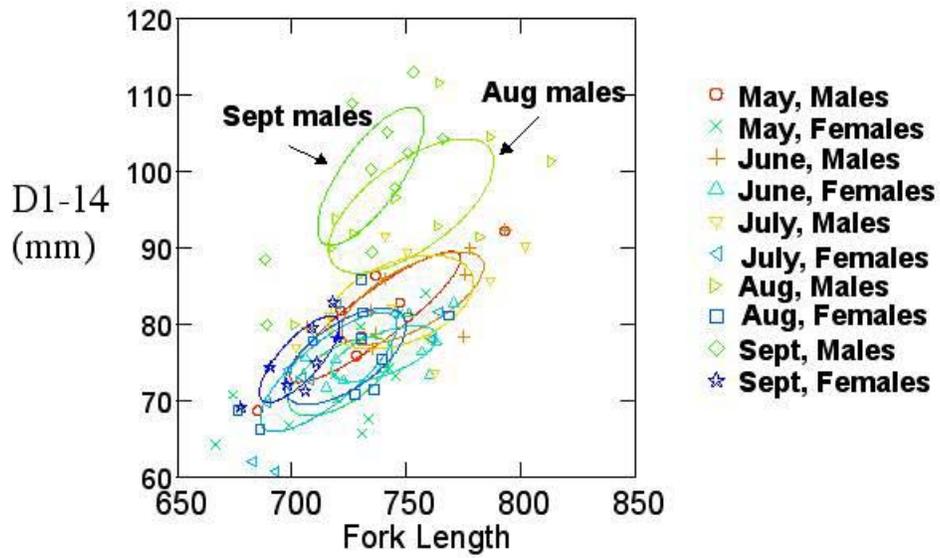


Figure 14. Scatter plot of D 1-14 on fork length (in mm) by month by sex with 95% confidence ellipses. Data are from Rapid River spring chinook salmon.

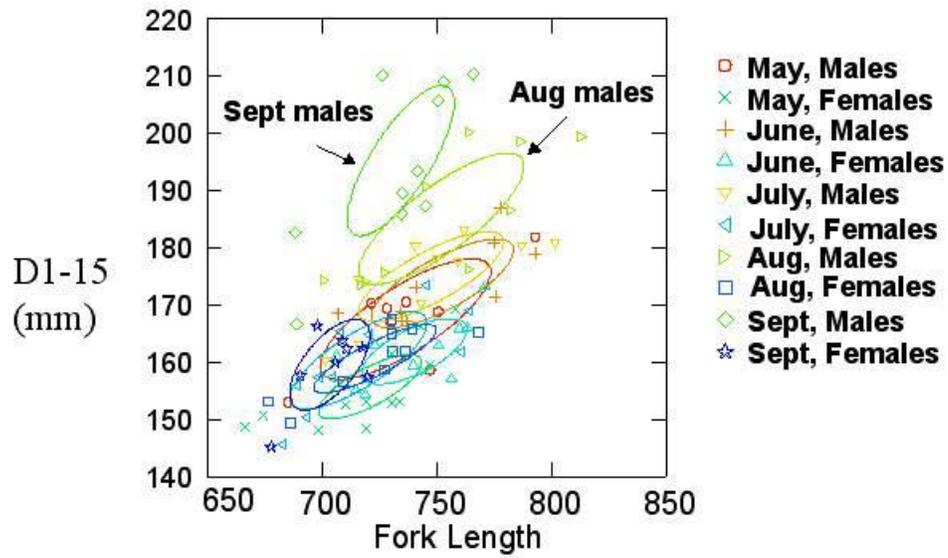


Figure 15. Scatter plot of D 1-15 on fork length (in mm) by month by sex with 95% confidence ellipses. Data are from Rapid River spring chinook salmon.

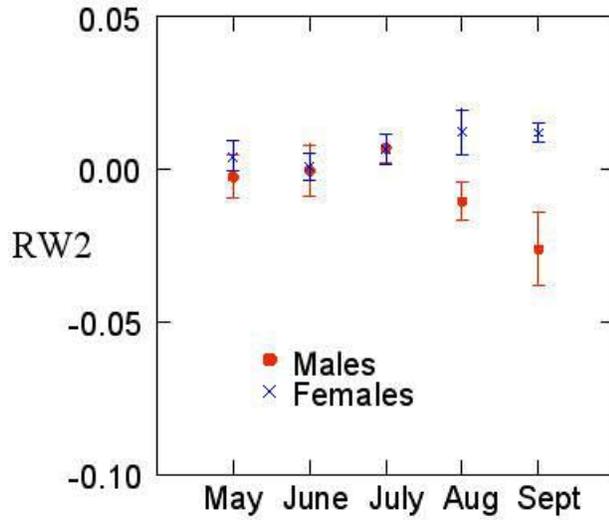


Figure 16. Mean relative warp (RW) scores for RW2 ($\pm 2SE$), $n=10$ /data point for Rapid River spring chinook salmon.

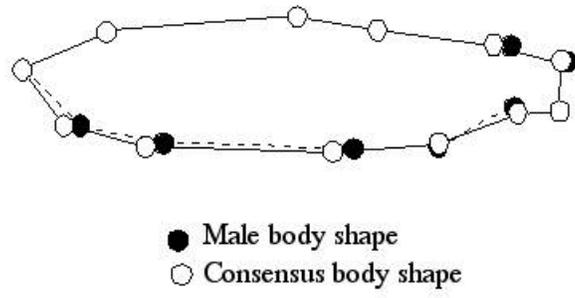


Figure 17. Shape changes in male Rapid River spring chinook salmon for RW2 with respect to the consensus configuration.

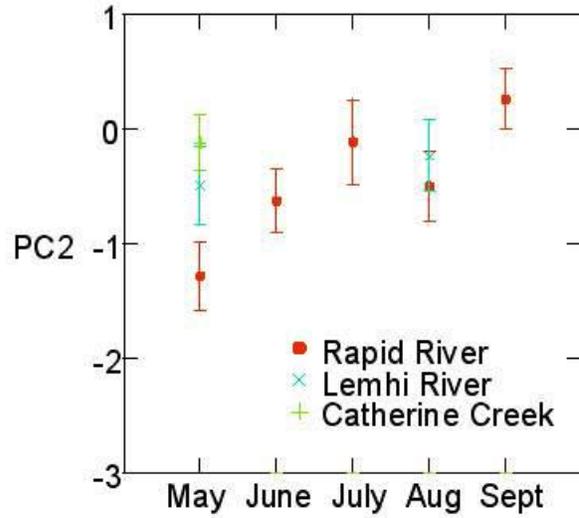


Figure 18. Distribution of principal component (PC) 2 scores ($\pm 2SE$), $n=5-10$ per date, for female spring chinook salmon from Lemhi and Catherine Creek captive broodstock in comparison to Rapid River females.

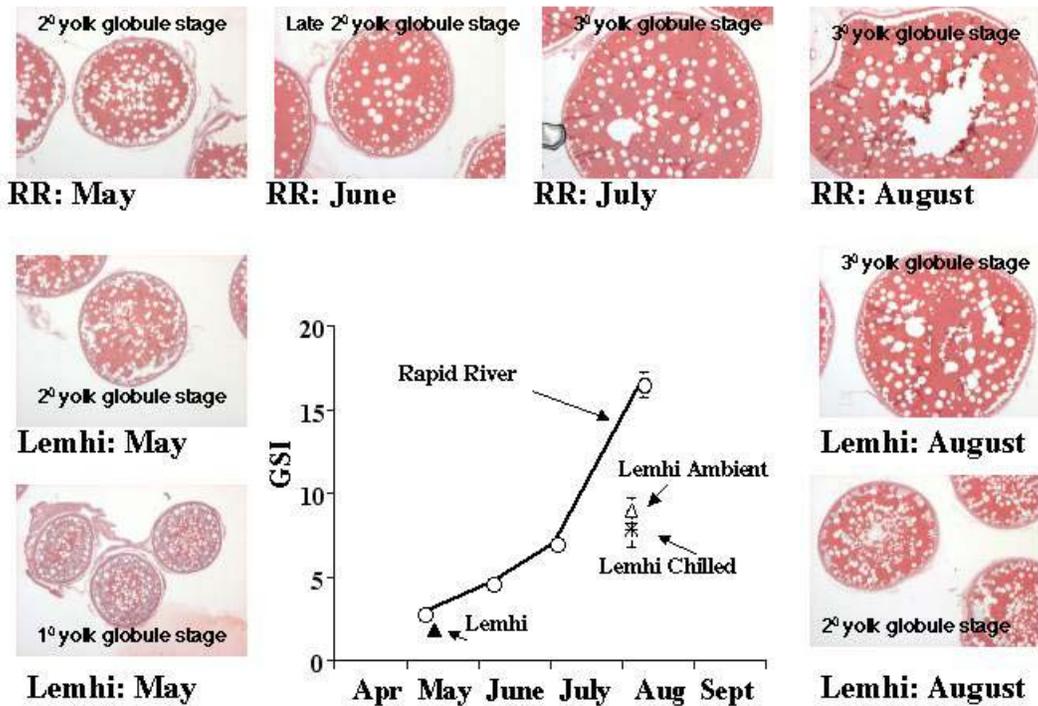


Figure 19. Changes in gonadosomatic indices (GSI) in Rapid River (RR) and Lemhi River spring chinook salmon females over the sampling period. Data are mean \pm SE, n=8-10. Error bars smaller than the data point are not shown. Representative examples of ovarian histology for each group at each time are shown in photographs. All photographs were taken at the same magnification; 20x. Individual data for Lemhi River fish are shown in Appendix I. Oocytes from the Rapid River females progressed from secondary to tertiary yolk globule stage from May to July. Oocytes from the Lemhi River females were more variable in stage, smaller in size and less advanced than the Rapid River females in May with some fish in the primary yolk globule stage.

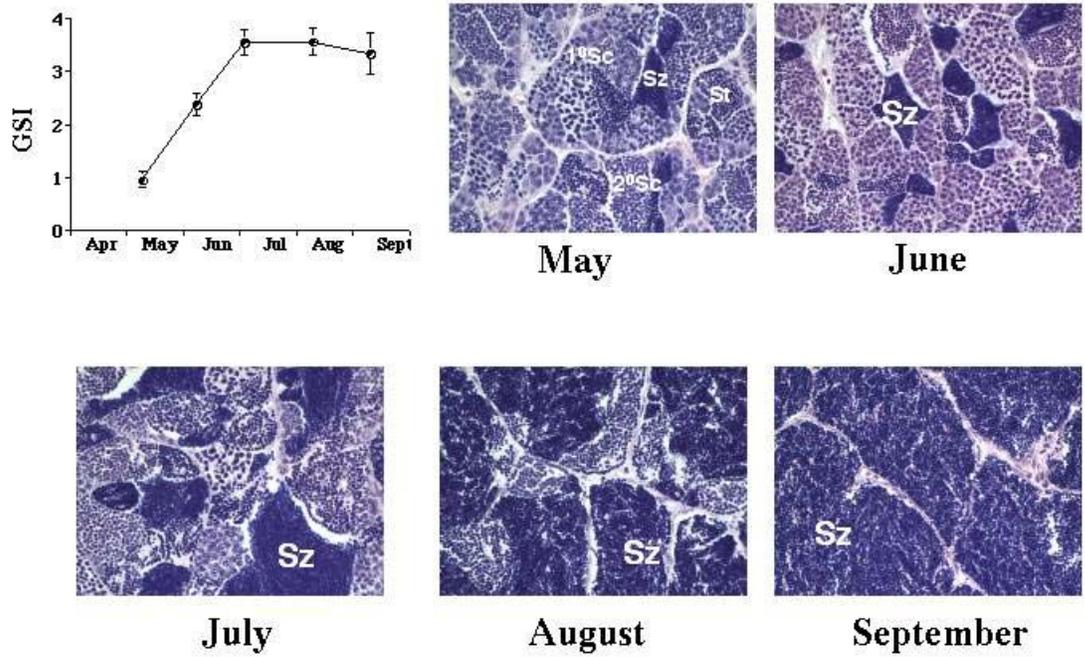


Figure 18. Changes in gonadosomatic indices (GSI) in Rapid River spring chinook salmon males over the sampling period. Data on GSIs are mean \pm SE, n=10/data point. Representative examples of ovarian histology for each group at each time are shown in photographs. All photographs were taken at the same magnification; 100x. Primary spermatocytes (10 Sc), secondary spermatocytes (20 Sc), spermatids (St) and spermatozoa (Sz) are indicated.

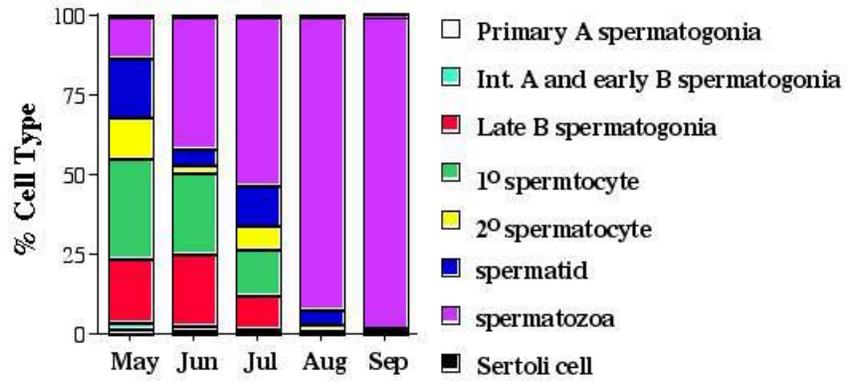


Figure 21. Percentage of cell types in the testes of male Rapid River spring chinook salmon sampled from May through September 2001. In September, males were spermiating and milt could be collect with gentle abdominal pressure. Data are means of 10 samples per month.

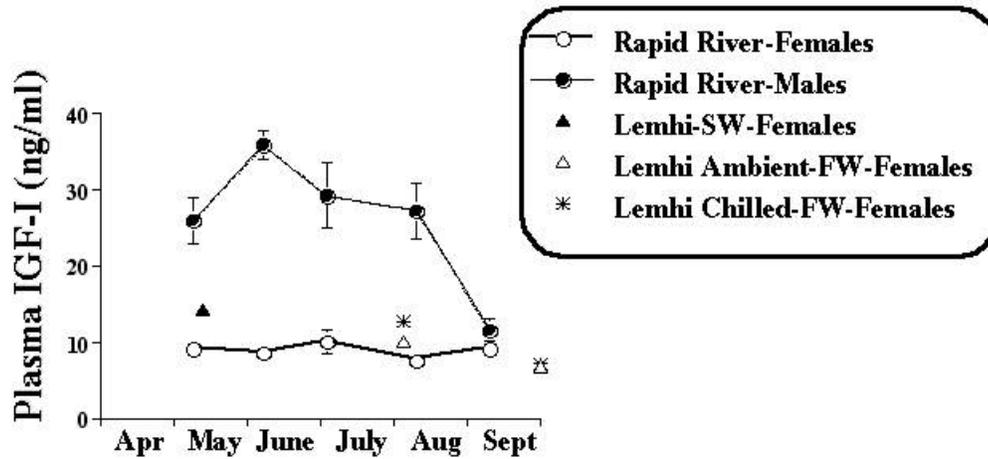


Figure 22. Plasma insulin like growth factor-I (IGF I) levels in Rapid River spring chinook salmon males and females, and Lemhi River spring chinook salmon females over the sampling period. Data are mean \pm SE, n=5-10. Error bars smaller than the data point are not shown. All Rapid River fish were in fresh water. SW, seawater. FW, fresh water. Ambient = 14⁰C, Chilled = 9⁰C.

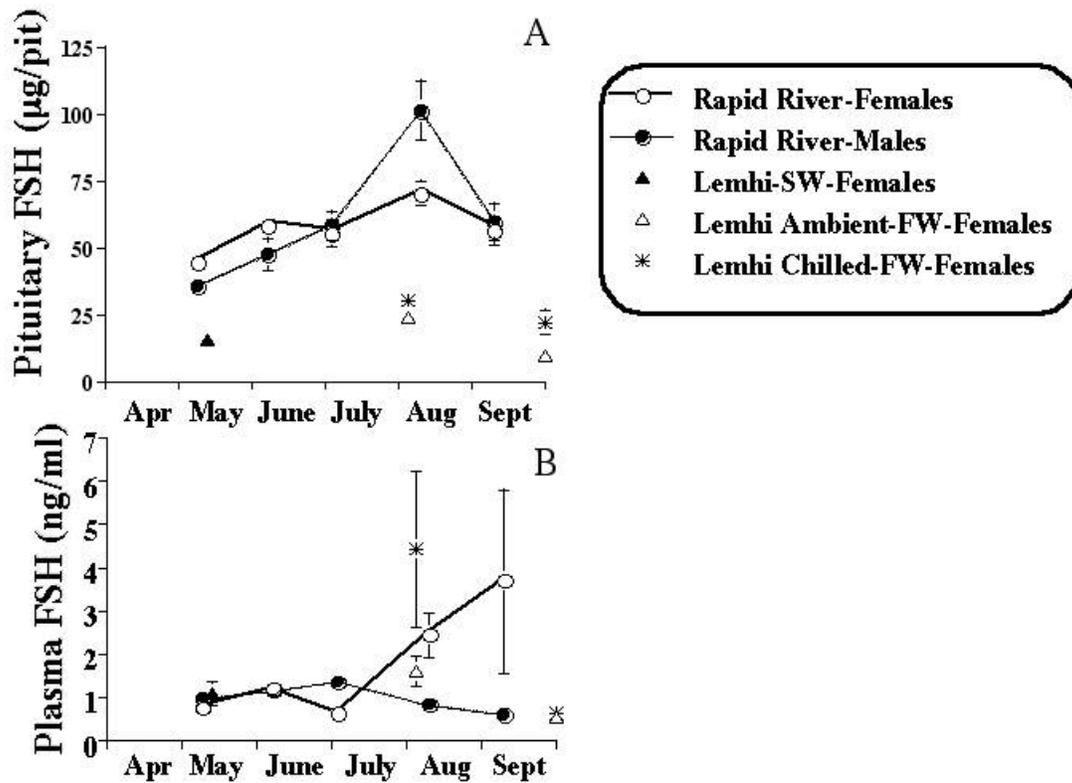


Figure 23. Pituitary (A) and plasma (B) follicle stimulating hormone (FSH) levels in Rapid River spring chinook salmon males and females, and Lemhi River spring chinook salmon females over the sampling period. Data are mean \pm SE, n=5-10. Error bars smaller than the data point are not shown. All Rapid River fish were in fresh water. SW, seawater. FW, fresh water. Ambient = 14⁰C, Chilled = 9⁰C.

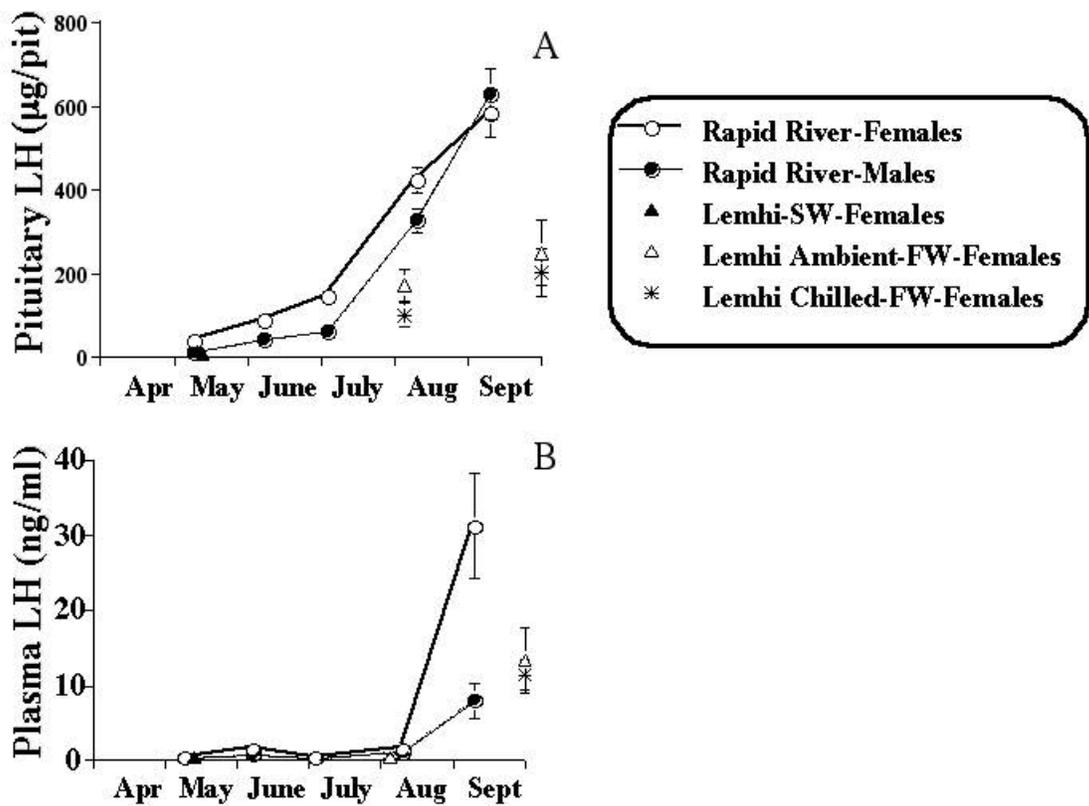


Figure 24. Pituitary (A) and plasma (B) luteinizing hormone (LH) levels in Rapid River spring chinook salmon males and females, and Lemhi River spring chinook salmon females over the sampling period. Data are mean \pm SE, n=5-10. Error bars smaller than the data point are not shown. All Rapid River fish were in fresh water. SW, seawater. FW, fresh water. Ambient = 14⁰C, Chilled = 9⁰C.

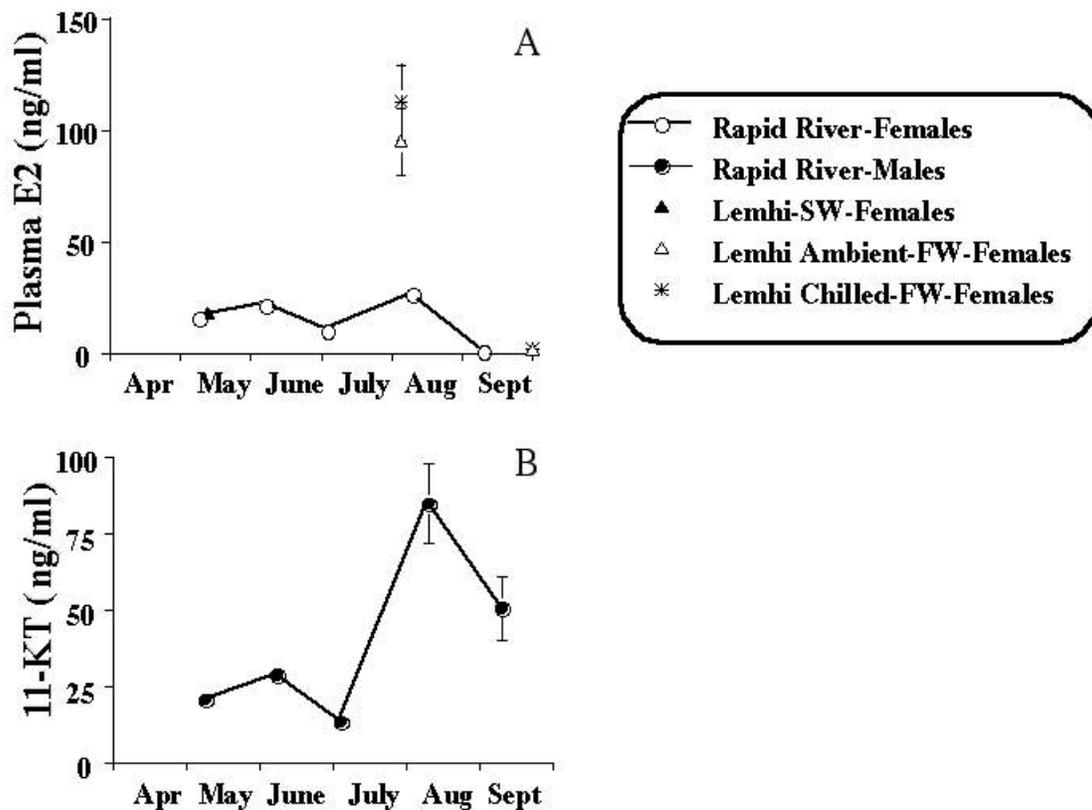


Figure 25. Plasma levels of estradiol-17 β (A) and 11-ketotestosterone (B) in Rapid River spring chinook salmon males (B) and females (A), and Lemhi River spring chinook salmon females (A) over the sampling period. Data are mean \pm SE, n=5-10. Error bars smaller than the data point are not shown. All Rapid River fish were in fresh water. SW, seawater. FW, fresh water. Ambient = 14 $^{\circ}$ C, Chilled = 9 $^{\circ}$ C.

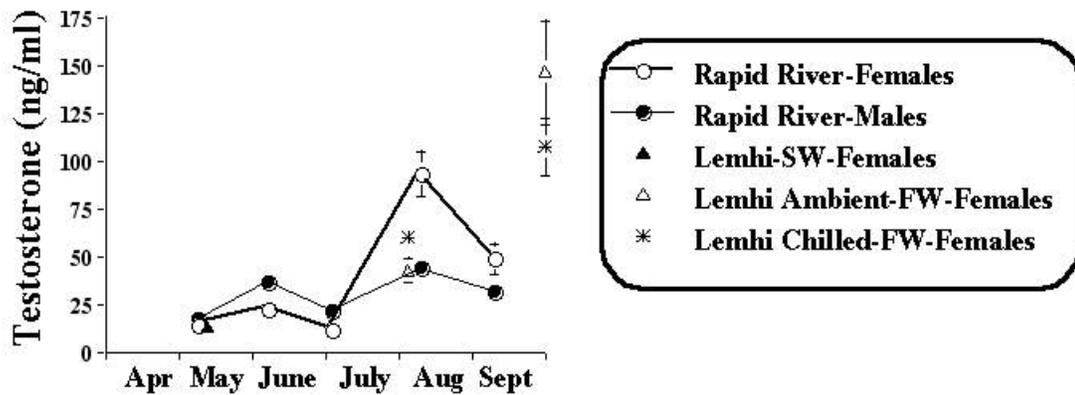


Figure 26. Plasma levels of testosterone in Rapid River spring chinook salmon males and females, and Lemhi River spring chinook salmon females over the sampling period. Data are mean \pm SE, n=5-10. Error bars smaller than the data point are not shown. All Rapid River fish were in fresh water. SW, seawater. FW, fresh water. Ambient = 14⁰C, Chilled = 9⁰C.

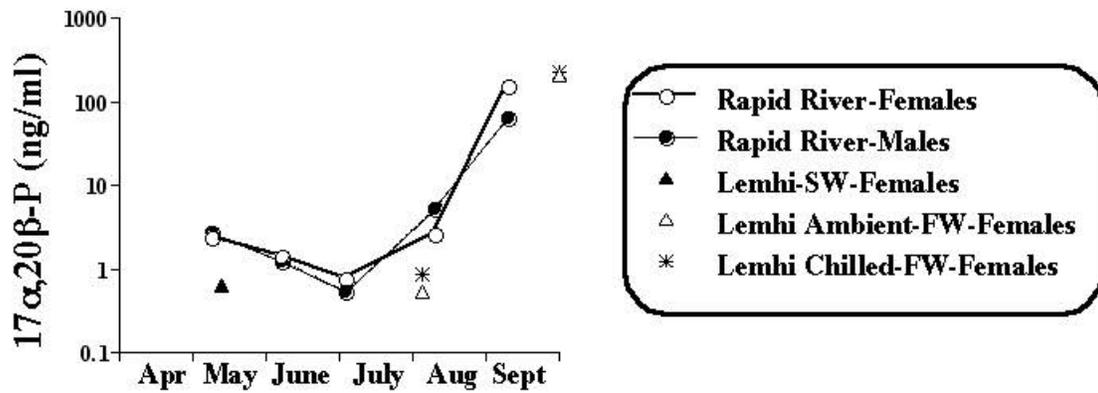


Figure 27. Plasma levels of 17 α ,20 β -dihydroxy-4-pregnen-3-one in Rapid River spring chinook salmon males and females, and Lemhi River spring chinook salmon females over the sampling period. Data are mean \pm SE, n=5-10. Error bars smaller than the data point are not shown. All Rapid River fish were in fresh water. SW, seawater. FW, fresh water. Ambient = 14 $^{\circ}$ C, Chilled = 9 $^{\circ}$ C.

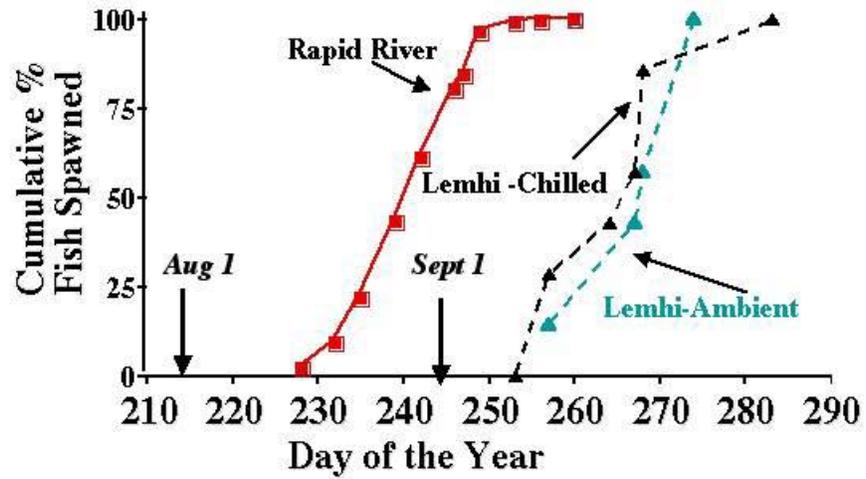


Figure 28. Cumulative percent of fish spawned over time in fall 2002. Data for Rapid River fish were provided by staff from the IDFG Rapid River Hatchery, Riggins, ID. Data for the Lemhi River females were obtained from NMFS-reared fish spawned at the IDFG Eagle Fish Hatchery, Eagle, ID. Ambient = 14⁰C, Chilled = 9⁰C.

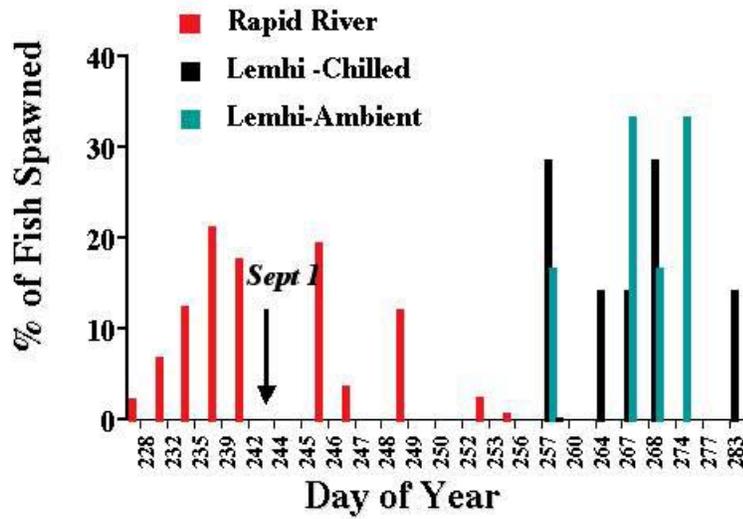


Figure 29. Percent of fish spawned over time in fall 2002. Data for Rapid River fish were kindly provided by staff from the IDFG Rapid River Hatchery. Data for the Lemhi River females were obtained from NMFS-reared fish spawned at the IDFG Eagle Fish Hatchery, Eagle, ID. Ambient = 14⁰C, Chilled = 9⁰C.

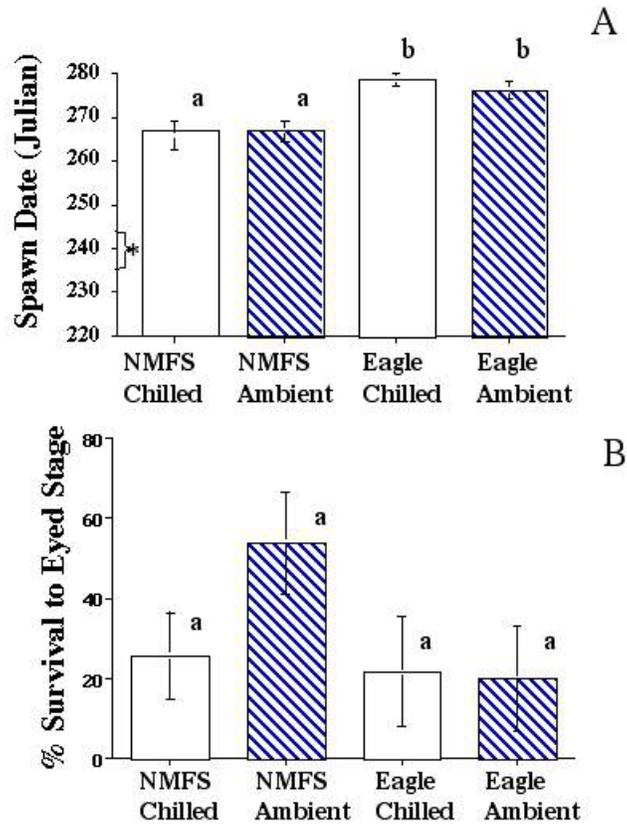


Figure 30. Mean days to spawning (A) and percent of eggs surviving to the eyed-stage in Lemhi River females reared from May 1999-June 2001 at either the NMFS Manchester Marine Research Station or the IDFG Eagle Fish Hatchery. From June 2001 to spawning in September and October 2001, groups of fish from each rearing site were reared on either chilled or ambient fresh water at the Eagle Fish Hatchery. Ambient = 14⁰C, Chilled = 9⁰C.

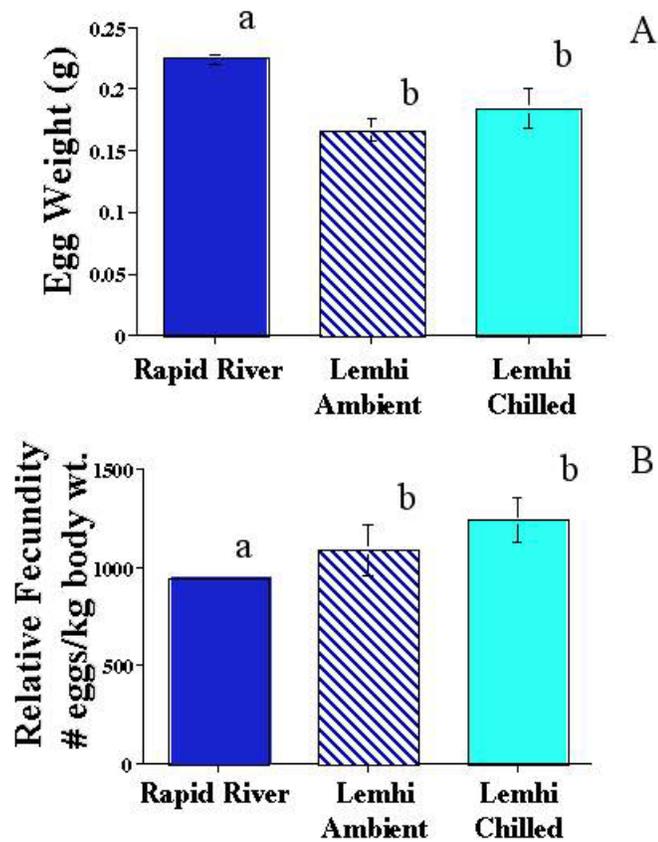


Figure 31. Egg weight (A) and relative fecundity (B) in returning Rapid River hatchery and captively-reared Lemhi River female spring chinook salmon (NMFS reared fish only). Data are mean \pm SE, n=10 Rapid River group; n= 5/ Lemhi River groups. Significant differences ($p < 0.05$) are indicated by different letters. Ambient = 14⁰C, Chilled = 9⁰C.

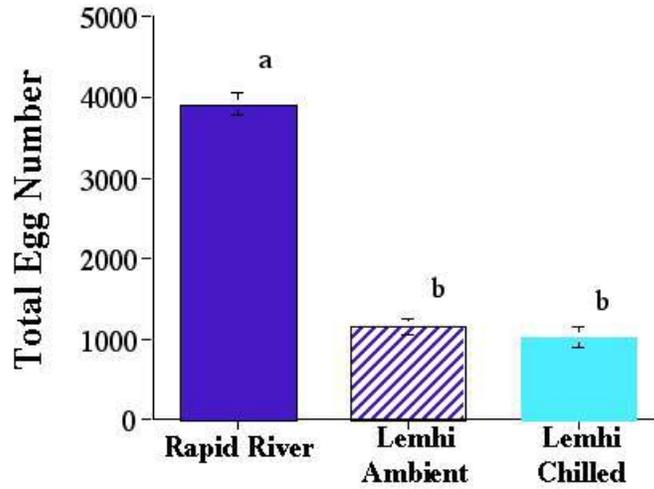
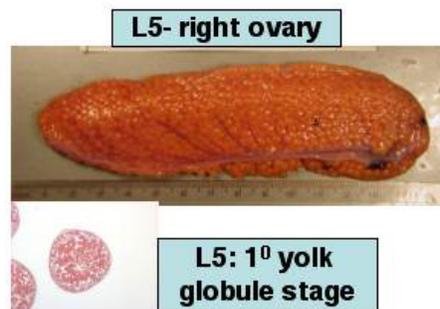
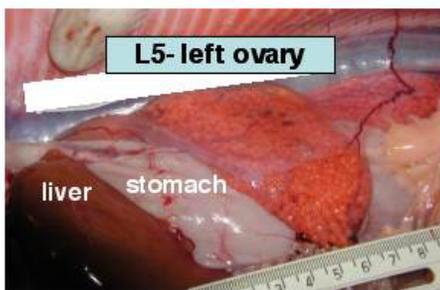
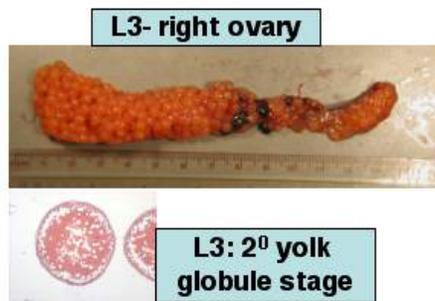
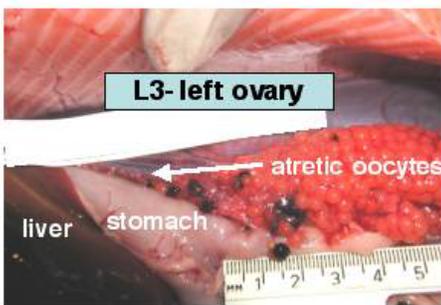
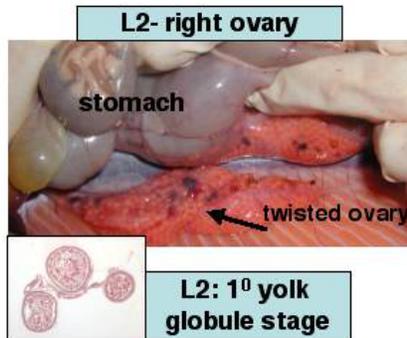
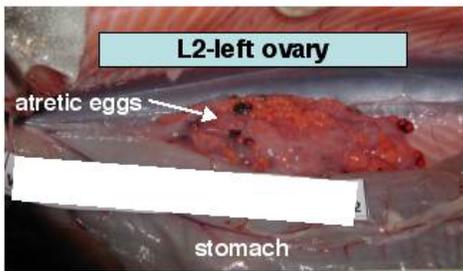
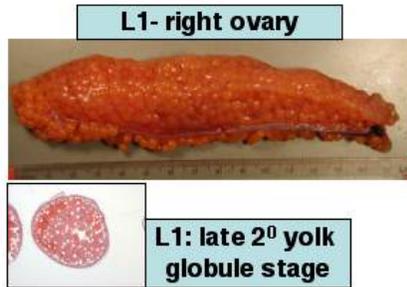
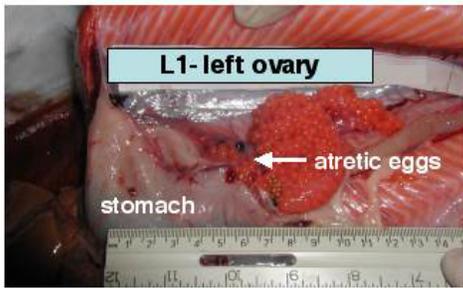
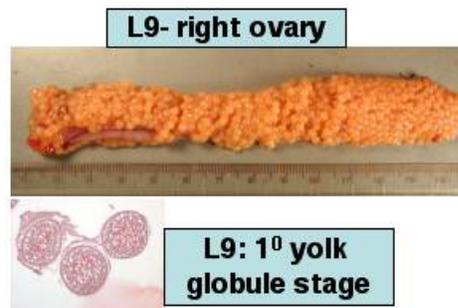
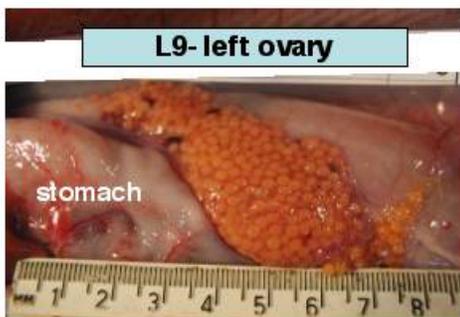
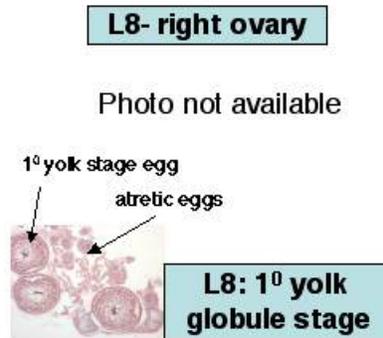
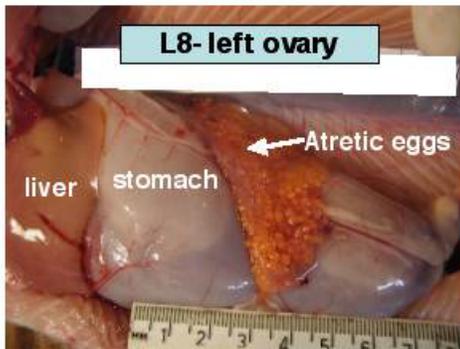
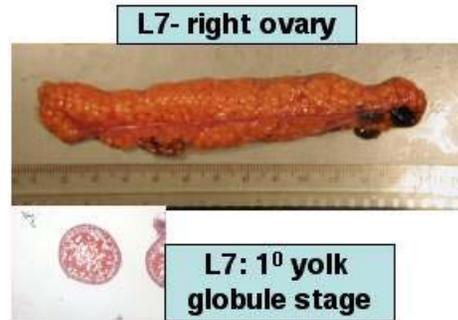
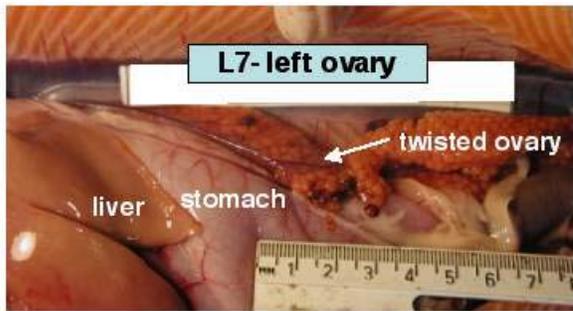
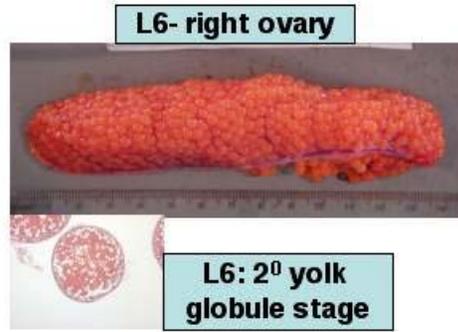
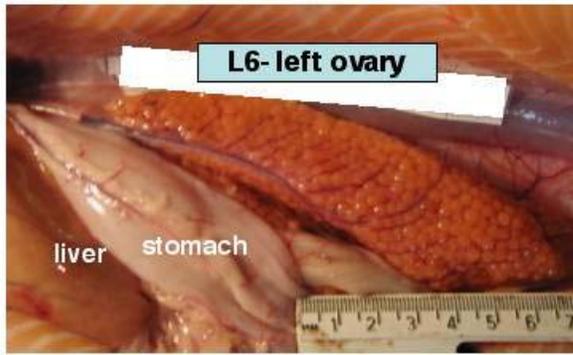


Figure 32. Total egg number in returning Rapid River hatchery and captively-reared Lemhi River female spring chinook salmon (NMFS reared fish only). Data are mean \pm SE, n=10 Rapid River group; n= 5/ Lemhi River groups. Significant differences ($p < 0.05$) are indicated by different letters. Ambient = 14⁰C, Chilled = 9⁰C.

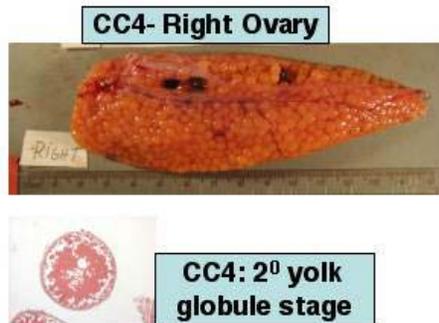
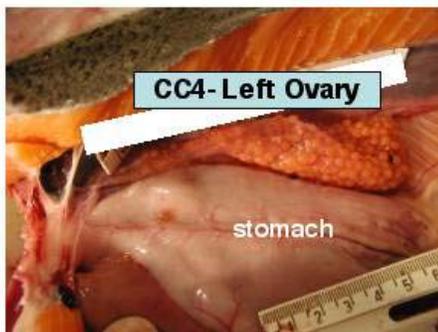
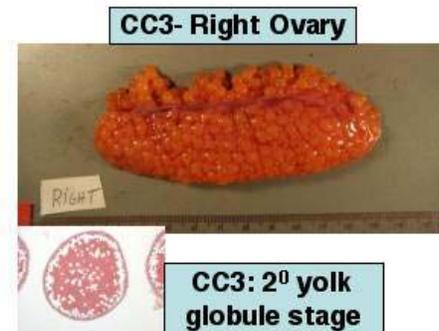
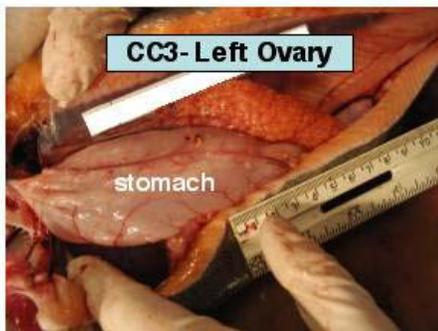
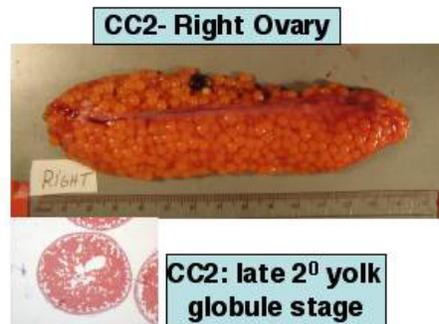
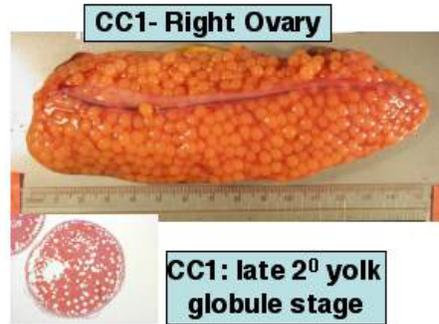
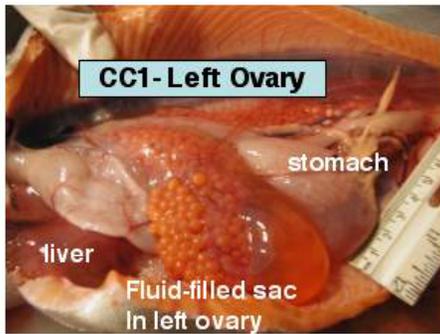
APPENDIX I

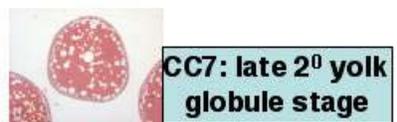
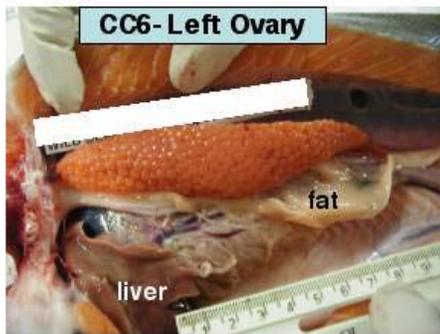
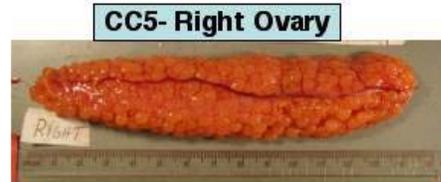
Photographs of ovaries and ovarian histology from samples (L1-L9) collected in May from Lemhi River spring chinook salmon reared at the NMFS Manchester Research Station.





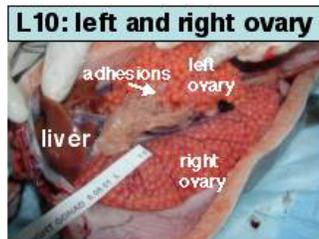
Photographs of ovaries and ovarian histology from samples collected in May 2001 from Catherine Creek spring chinook salmon reared in seawater (C1-5) at the NMFS Manchester Research Station or in fresh water (C6-10) at the Bonneville Hatchery.







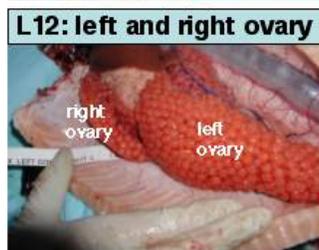
Photographs of ovaries and ovarian histology from samples collected in August 2001 from Lemhi River spring chinook salmon reared in seawater at the NMFS Manchester Research Station from May 1999 to June 2001, and then transferred to the IDFG Eagle Hatchery in June 2001 where they were maintained on either chilled or ambient fresh water until spawning in September and October 2001. Samples L11-L17 were from fish reared on chilled water. Samples L18-26 were from fish reared on ambient water. All photographs of gonad histology were taken in the same magnification.



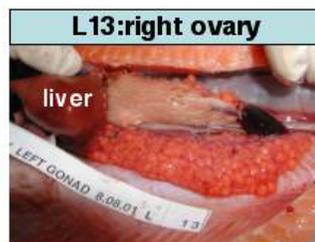
L10: 3⁰ yolk globule stage



L11: 3⁰ yolk globule stage



L12: 3⁰ yolk globule stage



L13: 2⁰ yolk globule stage



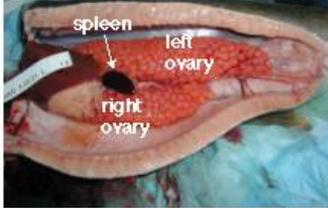
L14: early 3⁰ yolk globule stage



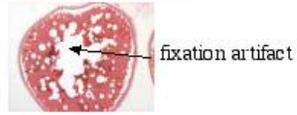
L15: early 3⁰ yolk globule stage



L16: left and right ovary



L16: early 3⁰ yolk globule stage



L17: left and right ovary



L17: early 3⁰ yolk globule stage



L18: left ovary



L18: early 3⁰ yolk globule stage



L19: left and right ovary



L19: adhesions to stomach



L19: early 3⁰ yolk globule stage



L20: left and right ovary



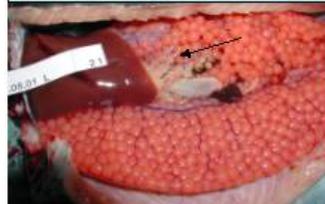
L20: early 3⁰ yolk globule stage



L21: left and right ovary



L21: adhesions to stomach

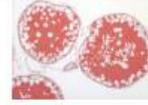


L21: early 3⁰ yolk globule stage





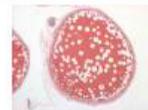
L22: late 2⁰ yolk globule stage



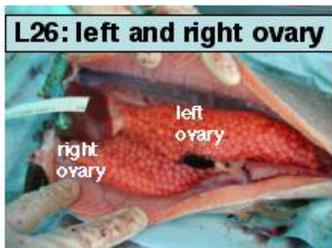
L23: early 3⁰ yolk globule stage



L24: late 2⁰ yolk globule stage



L25: early 3⁰ yolk globule stage



L26: early 3⁰ yolk globule stage



OBJECTIVE 3 - IMPROVE PHYSIOLOGICAL DEVELOPMENT AND MATURATION

TASK 7. EFFECTS OF WATER TEMPERATURE DURING THE SEAWATER REARING PHASE ON THE TIMING OF SPAWNING AND EGG QUALITY IN SPRING CHINOOK SALMON

By

Penny Swanson, Briony Campbell, Nicholas Hodges, Jon T. Dickey, Robert Endicott, and Barry J. Berejikian

Introduction

In captive broodstock programs for Snake River spring chinook salmon there are substantial problems with delayed spawning time and poor egg quality. The degree of the delay in spawning time relative to wild fish appears to be less for Oregon stocks (2-3 weeks) than with Idaho stocks (3-5 weeks). The delay in spawning time also appears to vary from year to year. It is not known to what degree the problem with delay in spawning and problems with egg fertility are related. The frequent occurrence of polarized over-ripe eggs suggests problems with final oocyte maturation and ovulation. Egg quality problems in rainbow trout have been attributed to inappropriate timing of egg collection relative to ovulation (Springate et al. 1984), thus improving the timing of ovulation may help to improve egg quality in the spring chinook salmon captive broodstock.

It is well known that the seasonal timing of spawning in salmonids is primarily regulated by photoperiod and temperature (Bromage 1995, Bromage et al. 1993). Photoperiod has been successfully used to alter the time of spawning in a number of salmonids. However, this is not presently feasible in captive broodstock programs because each group is often a mixture of maturing and non-maturing fish, and photoperiod cannot be used to advance spawn time of the maturing fish without interfering in the spawning of the non-maturing fish during the following year. Until reliable methods are available to sort fish by maturity early in the year and sufficient facilities are available to rear maturing and non-maturing fish separately, the use of photoperiod to synchronize and advance spawning of broodstock will not be practical. Spawn timing has also been advanced using implants containing hormone analogues, such as gonadotropin-releasing hormone (Swanson 1995). The use of this drug is not permitted for released fish, but it is being used successfully to advance captive-spawned fish on a 'as needed' basis.

There is growing evidence that rearing temperatures can alter the timing of spawning of salmonids (Henderson 1963, Goryczko 1972, Titarev 1975, Morrison and Smith 1986, Nakari et al. 1987 and 1988). High water temperatures have been shown to inhibit the later stages of maturation in salmonids (for rainbow trout see Billard and Breton 1977,

Breton et al. 1983, Pankhurst et al. 1996, Pankhurst and Thomas 1998, Chmilevsky 1999, Davies and Bromage 2002; for pink salmon see Beacham and Murray 1988; for Atlantic salmon see Johnstone et al. 1992, Taranger and Hansen 1993, Taranger et al. 1999, King and Pankhurst 1999; for Arctic charr see Gillet 1991, Jobling et al. 1995) and can cause egg over-ripening before or after ovulation with corresponding effects on embryogenesis and fry survival.

Studies have shown that, to maximize survival of offspring, it is essential that eggs undergo development and are ovulated or artificially stripped at optimum temperatures (e.g., Billard and Breton 1977, Billard and Gillet 1981, Taranger and Hansen 1993, Pankhurst et al. 1996, Pankhurst and Thomas 1998, Davies and Bromage 2002). A number of these studies have also reported ovarian dysfunction and poor egg quality associated with high temperatures during maturation. For example, Pankhurst et al. 1996 described the occurrence of unovulated eggs bearing a resemblance to over-ripe eggs in rainbow trout exposed to constant water temperatures of 18 and 21°C during final maturation, a phenomenon confirmed by Davies and Bromage (2002). In addition, relatively moderate elevations in temperature (only a few degrees difference) have been shown to reduce significantly egg fertility and survival to eyed (e.g., Pankhurst et al. 1996, Taranger and Hansen 1993).

Two key differences in three captive broodstock rearing facilities for Snake River spring chinook salmon (Eagle Fish Hatchery ID, Bonneville Hatchery OR, and the Manchester Research Station WA) are the type of water (fresh versus saltwater) and rearing water temperature. Ambient water temperature at Eagle Hatchery is generally constant at 14°C, unless water is chilled while at the Bonneville Hatchery water temperatures are relatively constant at 9°C. At Manchester, seawater temperatures range from 9°C in January to 13.5°C in August. Given the limitations of broodstock facilities with regard to temperature control of fresh water and the expense of chilling water, it is critical to determine if water temperature is one of the underlying causes of delayed maturation and poor egg quality.

It is impractical to determine optimal spawning temperature for all stocks in captive broodstock programs. A previous study suggested that rearing environment prior to transfer to adults to fresh water for spawning affected spawn time (see Task 6). Therefore, an experiment to determine if reducing temperature during the seawater rearing stage can alter spawning time and fertility of eggs in Carson spring chinook salmon is being conducted. The temperature ranges that more closely resemble temperatures experienced in the ocean phase of the life cycle are being tested.

Materials, Methods, and Description of Study Area

The hypothesis of the study is that reduced water temperature during the 7 months of saltwater rearing prior to spawning does not alter reproductive performance (timing of spawning, fecundity, fertility, behavior). The overall design of this experiment is to divide 3-year old Carson spring chinook salmon into two treatment groups (2 tanks per

treatment) which differ in water temperature profiles during the 7 months of rearing in saltwater (October 2002-April 2003), and then transfer fish in May 2003 to a common freshwater environment maintained at a constant 10-11°C until spawning in August-September 2003.

Fish were tagged with PIT tags during July 2002, sexed with a genetic sex marker (Du et al. 1993), and are being reared in fiberglass tanks in filtered and UV treated saltwater at Manchester. Two temperatures are being compared: ambient (low of 10°C in January, high of 13°C in August), and chilled (low of 4 °C in January, and high of 9°C in August). Fish are being fed Moore Clark Pedigree Salmon Brood Diet and a standard ration adjusted for rearing temperature until late April 2003. During July and September 2002, February and May 2003 body size of all tagged fish was determined and 3 females per tank were sacrificed to assess ovarian development.

Samples of plasma, pituitary glands and ovarian tissue were collected from the sacrificed fish. Plasma will be analyzed for sex steroids and gonadotropins once all samples are collected at spawning. Ovarian tissue was fixed in Bouin's solution for subsequent histological analyses. Carcasses, viscera and ovaries were frozen for proximate analyses. During May 2003, body size was measured and fish were transferred to fresh water and food has been withdrawn. Samples for hormone analyses will not be analyzed until final blood samples are collected at spawning in August and September 2003. Ovarian tissue has been processed for histological analyses.

Results and Discussion

As of February 2003 there was no difference in average size of fish between the treatments (Figure 1).

Variation in ovarian stage was observed in the terminally sampled fish (Figures 2 and 3). Smaller fish tended to be less advanced than larger fish; however, because of the low sample number for the terminal samples and large variance in size and stage, it is not possible to determine at this point if there are treatment effects on development that are independent of body size. In this analysis only samples of healthy eggs were processed for histological analysis.

During rearing of fish for this experiment, one of the major problems encountered was severe chronic gastric distention which appears to cause compression atrophy of the left ovary. This was observed in 50-70% of the females sampled at all sampling times. Examples of this disorder are shown in Figures 4 and 5. The ratio of the weight of the left to the right ovary is an index of the degree of regression of the left ovary (see Figures 2 and 3). The cause of this disorder has not been determined; however, at least one report in the literature suggested that a component of the diet which affects digestibility or gut motility may be causing this problem (Lumsden et al. 2002). It is not known to what degree this problem will affect the outcome of this experiment, but there were no apparent treatment differences in the incidence of the gastric distention.

Commencing in late August 2003, fish will be checked manually at weekly intervals for maturity. Once the first ovulated female is detected, fish will be checked every 3-4 days. This frequency of handling fish is necessary to identify the timing of ovulation as precisely as possible without inducing excessive handling stress.

At spawning, body weight and length, total egg weight, egg number, and individual egg weights will be measured. Fertility and survival of embryos will be assessed at several spawn dates to determine treatment effects on egg quality. The numbers of females assessed at each time will depend on timing of spawning and if there are any treatment effects on spawn date. A total of 3 females per tank (6 per treatment) will be analyzed on two spawn dates.

Eggs will be divided into 2 lots of 200, and fertilized with milt from a pool of 3-6 males. Because of a shortage of 4-year old maturing males, it is necessary to use pooled milt. Milt from all males will be examined for motility. Non-motile sperm will be discarded. Fertilized eggs will be incubated at the Montlake Laboratory in Heath Trays (4 inch diameter cups for each cross) at 9°C. Fertilization rates will be determined by clearing sub samples of 50 eggs per cross in Stockard's solution and examining each egg for the presence of first or second cleavage (samples collected within 24 hrs of fertilization). Survival to blastopore closure and the eyed-stage will be monitored by collecting sub samples of eggs at approximately 16 and 30 days post fertilization. At spawning, 3 batches of 200 ovulated eggs will also be photographed to document presence of polarized, over-ripe eggs.

Treatment effects on all parameters (body size, composition, fertility, fecundity, egg size and spawn date) will be determined by ANOVA. Spawning carcasses will be analyzed for proximate composition to determine temperature effects on mobilization of energy reserves. Spawning behavior and reproductive success in the experimental spawning channel will be monitored in 8 females/tank under Objective 1. It is expected that the number of replicate females per spawning date will vary, but it is anticipated that a minimum of 2-3 females per tank per treatment per spawn date will be available for the manual spawning phase of the experiment.

Data Management Activities

Data are collected manually by NOAA, UW, and PSMFC researchers onto preformatted data sheets or directly into PCs. Data are entered and summarized on personal computers operated by researchers using Microsoft Excel, Statview 512+, Cricket Graph 1.3.2, and Prism. Data on histology of tissues is recorded using a digital camera and stored as either TIFF or JPG files on a Power MacIntosh G4. Backup copies of data are saved on an external Lacie hard-drive and on CD-ROMs. All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report under Materials and Methods. Data analyses are reported in the Results section of the annual report.

Summary and Conclusions

Not applicable at this time.

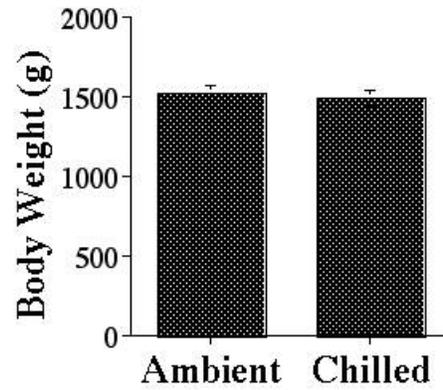


Figure 1. Average body weight of Carson spring chinook salmon females on February 11, 2003. Fish are being reared in either ambient or chilled seawater from October 2002 to May 2003. Data are mean \pm S.E. of two replicate tanks. Each tank contained 34 to 37 females. There were no significant treatment differences in either body weight or length (data not shown).

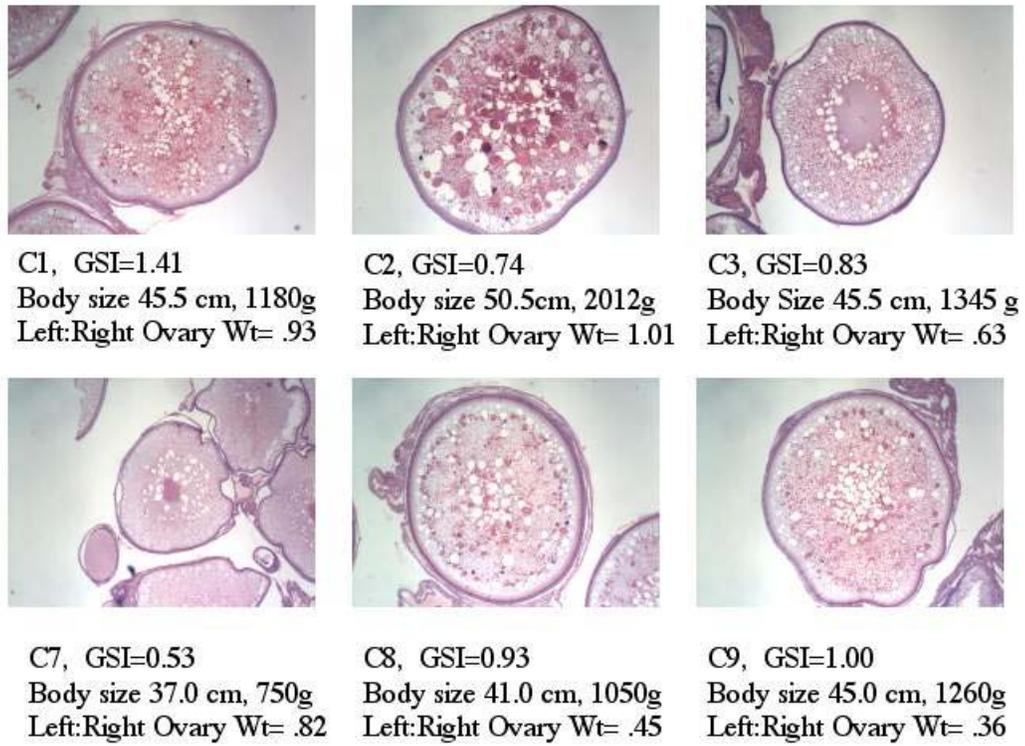
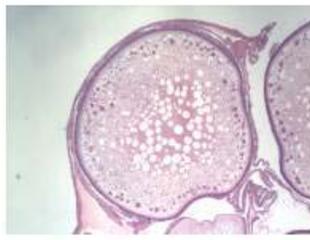


Figure 2. Ovarian histology, body size, and ratio of left to right ovary weight of fish sampled in February, 2002 from Ambient group (3 fish sampled per tank). Ovaries from fish numbers C1, C3, C8, and C9 were in very early stages of primary yolk stage while that of C3 and C7 were in the lipid droplet stage that precedes yolk incorporation. The ovary of fish C2 was in the primary yolk stage.



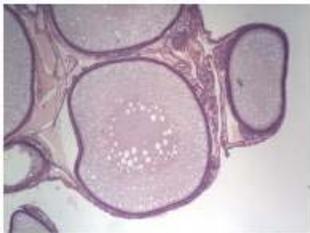
C4, GSI=0.77
 Body size 45.8cm, 1460g
 Left:Right Ovary Wt= .88



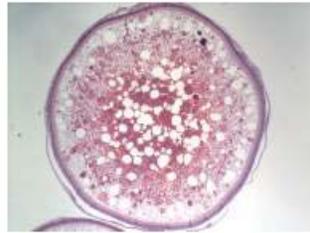
C5, GSI=0.77
 Body Size 48.5cm, 2060g
 Left:Right Ovary Wt= 1.03



C6, GSI=1.03
 Body size, 48.5 cm, 1780g
 Left:Right Ovary Wt= 1.01



C10, GSI=0.65
 Body size, 43.5cm, 1059g
 Left:Right Ovary Wt= .56



C11, GSI=0.83
 Body size, 45.5cm, 1408g
 Left:Right Ovary Wt= .46



C12. GSI=0.83
 Body size, 45.8cm, 1495g
 Left:Right Ovary Wt= .61

Figure 3. Ovarian histology, body size, and ratio of left to right ovary weight of fish sampled in February, 2002 from Chilled group (3 fish sampled per tank). Fish with ovary weight ratios of less than one showed regression of left ovary associated with gastric distention disorder. Ovaries from fish C4, C6, C11, and C12 were in varying degrees of advancement in the primary yolk stage while that of C5 was in the early primary yolk stage and C10 was in the lipid droplet stage that precedes yolk incorporation.

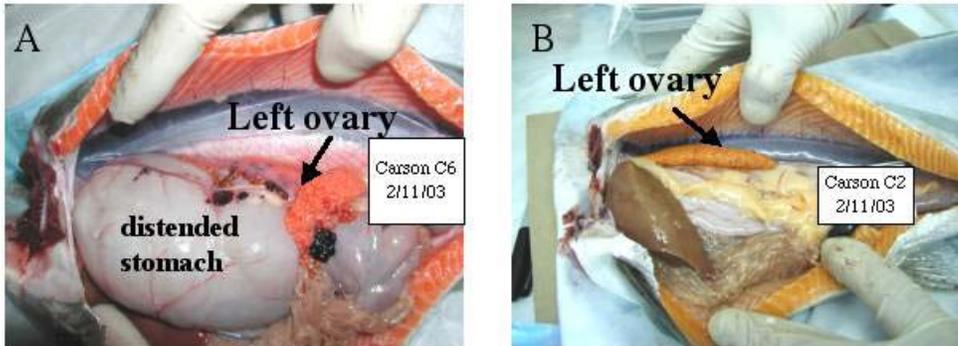


Figure 4. Left ovaries of female spring chinook salmon sampled on February 11, 2003. (A) Fish with gastric distention disorder and necrotic left ovary. Note that the ovary is twisted and contains hemorrhaging and atretic oocytes. The right ovary of this fish was normal. (B) Fish with normal left and right ovaries and no gastric distention. Only 2 out of 12 fish sampled had normal left and right ovaries. The remaining fish had varying degrees of gastric distention and necrosis of the left ovary.

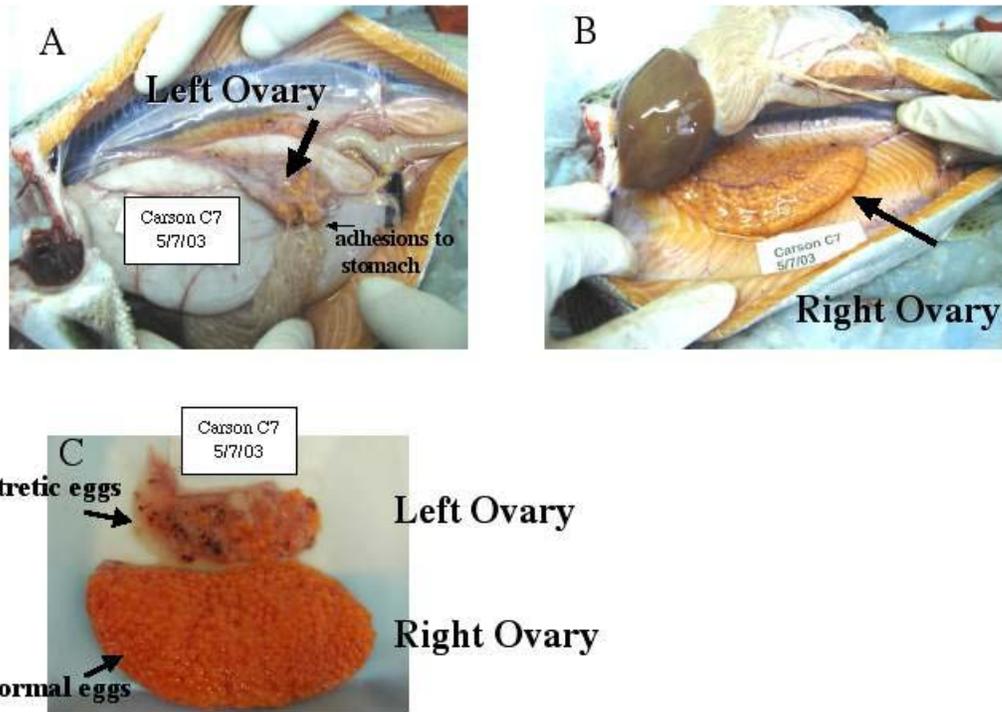


Figure 5. Left and right ovaries of one female (C7) spring chinook salmon sampled on May 7, 2003. (A) Left ovary is almost completely regressed and has many adhesions to the stomach. (B) Right ovary is normal. (C) Comparison of left and right ovaries.

References

- Beacham, T.D., and C.B. Murray. 1988. Influence of photoperiod and temperature on timing of sexual maturity of pink salmon (*Oncorhynchus gorbuscha*). *Can. J. Zool.* 66:1729-1732.
- Billard, R., and B. Breton. 1977. Sensibilité à la température des différentes étapes de la reproduction chez la truite arc-en-ciel. *Cah. Lab. Hydrobiol. Montereau* 5:5-24.
- Billard, R., and C. Gillet. 1981. Vieillessement des ovules et potentialisation par la température des effets des micropolluants du milieu aqueux sur les gametes chez la truite. *Cah. Lab. Hydrobiol. Montereau* 12:35-42.
- Breton, B., A. Fostier, Y. Zohar, P.Y. Le Bail, and R. Billard. 1983. Gonadotropine glycoproteique maturante et oestradiol-17 β pendant le cycle reproducteur chez la truite fario (*Salmo trutta*) femelle. *Gen. Comp. Endocrinol.* 49:220-231.
- Bromage, N. 1995. Broodstock management and seed quality-general considerations, pp. 1-24. *In* N.R. Bromage and R.J. Roberts (editors). *Broodstock Management and Egg and Larval Quality in Aquaculture*. Blackwell Science, London.
- Bromage, N., C. Randall, J. Duston, M. Thrush, and J. Jones. 1993. Environmental control of reproduction in salmonids, pp. 55-66. *In* J.F. Muir and R.J. Roberts (editors), *Recent Advances in Aquaculture* (Vol. 4). Blackwell Science, London.
- Brooks, S., C.R. Tyler, and J. P. Sumpter. 1997. Egg quality in fish: what makes a good egg? *Rev. Fish Biol. Fish.* 7:387-416.
- Chmielevsky, D.A. 1999. Effects of extreme ranged temperatures on fish oogenesis. *Proc. 6th International Symposium on the Reproductive Physiology of Fish*, July 4-9, 1999, p.316. University of Bergen, Norway.
- Davies, B., and N. Bromage. 2002. The effects of fluctuating seasonal and constant water temperatures on the photoperiodic advancement of reproduction in female rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 205:183-200.
- Du S.J., R.H. Devlin, and C.L. Hew. 1993. Genomic structure of growth hormone genes in chinook salmon (*Oncorhynchus tshawytscha*): presence of two functional genes, GH-I and GH-II, and a male-specific pseudogene, GH-y. *DNA and Cell Biol.* 12:739-751.
- Gillet, C. 1991. Egg production in an Arctic charr (*Salvelinus alpinus* L.) brood stock: effects of temperature on the timing of spawning and the quality of eggs. *Aquat. Living Resour.* 4:109-116.
- Goryczko, K. 1972. A change of the spawning season in rainbow trout (*Salmo gairdneri* Richardson). *Rocz. Nauk rol.* 94H:57-68.
- Henderson, N.E. 1963. Influence of light and temperature on the reproductive cycle of the eastern brook trout, *Salvelinus fontinalis* (Mitchell). *J. Fish Res. Board Can.* 20:859-897.
- Jobling, M., H.K. Johnsen, G.W. Pettersen, and R.J. Henderson. 1995. Effect of temperature on reproductive development in Arctic charr, *Salvelinus alpinus* (L.). *J. Therm. Biol.* 20:157-165.
- Johnstone, C.E., M. Hambrook, R.W. Gray, and K.G. Davidson. 1992. Manipulation of reproductive function in Atlantic salmon (*Salmo salar*) kelts with controlled photoperiod and temperature. *Can. J. Fish. Aquat. Sci.* 49:2055-2061.

- King, H.R., and N.W. Pankhurst. 1999. Ovulation of Tasmanian Atlantic salmon maintained at elevated temperatures: implications of climate change for sustainable industry development. *In Proc. 6th International Symposium on the Reproductive Physiology of Fish*, July 4-9, 1999, p.396-398. University of Bergen, Norway.
- Lumsen, J.S., P. Clark, S. Hawthorn, M. Minamikawa, S.G. Fenwick, M. Haycock, and B. Wybourne. 2002. Gastric dilation and air sacculitis in farmed chinook salmon, *Oncorhynchus tshawytscha* (Walbaum). *J. Fish Diseases* 25:155-163.
- Morrison, J.K. and C.E. Smith. 1986. Altering the spawning of rainbow trout by manipulating water temperature. *Prog. Fish-Cult.* 48:52-54.
- Nakari, T., A. Soivio, and S. Pesonen. 1987. Effects of an advanced photoperiod cycle on the gonadal development and spawning time of 2-year-old *Salmo gairdneri* R. reared in earth ponds under extreme annual water temperatures. *Aquaculture* 67:369-384.
- Nakari, T., A. Soivio, and S. Pesonen. 1988. The ovarian development and spawning time of *Salmo gairdneri* R. reared in advanced and delayed annual photoperiod cycles at naturally fluctuating water temperatures in Finland. *Ann. Zool. Fennici* 25:335-340.
- Pankhurst, N.W., G.J. Purser, G. Van Der Kraak, P.M. Thomas, and G.N.R. Forteach. 1996. Effect of holding temperature on ovulation, egg fertility, plasma levels of reproductive hormones and in vitro ovarian steroidogenesis in the rainbow trout *Oncorhynchus mykiss*. *Aquaculture* 146:278-290.
- Pankhurst, N.W., and P.M. Thomas. 1998. Maintenance at elevated temperature delays the steroidogenic and ovulatory responsiveness of rainbow trout *Oncorhynchus mykiss* to luteinizing hormone releasing hormone analogue. *Aquaculture* 166:163-177.
- Springate, J.R.C., N.R. Bromage, J.A.K. Elliott, and D.L. Hudson. 1984. The timing of ovulation and stripping and their effects on the rates of fertilization and survival to eyeing, hatching and swim-up in the rainbow trout (*Salmo gairdneri* R.). *Aquaculture* 42:313-322.
- Swanson, P., B.R. Beckman, B. Campbell, R. Campbell, K. Cooper, J.T. Dickey, N. Hodges, P. Kline, D.A. Larsen, K.D. Shearer, D. Venditti, and G. Young. 2002. Reproductive physiology of adult spring chinook salmon during five months preceding spawning. *In B.A. Berejikian and C.E. Nash (editors), Research on Captive Broodstock Programs for Pacific Salmon. Annual Report to Bonneville Power Administration for FY01.*
- Taranger, G.L., and T. Hansen. 1993. Ovulation and egg survival following exposure of Atlantic salmon, *Salmo salar* L., broodstock to different water temperatures. *Aquacult. Fish. Manage.* 24:151-156.
- Taranger, G.L., S.O. Stefansson, F. Oppedal, E. Andersson, T. Hansen, and B. Norberg. 1999. Photoperiod and temperature affects gonadal development and spawning time in Atlantic salmon (*Salmo salar* L.). *In Proc. 6th International Symposium on the Reproductive Physiology of Fish*, July 4-9, 1999, p.345. University of Bergen, Norway.
- Titarev, Y.F. 1975. Acceleration of maturation in rainbow trout (*Salmo gairdneri*) under the influence of increased water temperature. *J. Ichthyol.* 15:507-512.

OBJECTIVE 4 - IMPROVE IN-CULTURE SURVIVAL OF JUVENILES: PREVENTION AND CONTROL OF DISEASE

TASK 8. DEVELOPMENT AND TESTING OF VACCINES AND CHEMOTHERAPEUTANTS TO REDUCE MORTALITY FROM BACTERIAL KIDNEY DISEASE (BKD) IN CHINOOK SALMON

By

Linda D. Rhodes, Cindra K. Rathbone, Lee W. Harrel, Rebecca K. Andrews, and Mark S. Strom

Introduction

Bacterial kidney disease (BKD) is a major infectious disease that is problematic for successful culture of salmonids in the Pacific Northwest. BKD epizootics continue to significantly impact captive rearing programs of both sockeye and chinook salmon (Schiewe et al., 1997). There are no highly efficacious vaccines available to protect salmon from infections with *Renibacterium salmoninarum*, the causative agent of BKD. Between 1993-94 this disease was responsible for catastrophic losses of endangered Redfish Lake sockeye salmon held as captive broodstock. Epizootics of BKD in almost all captive broodstock and captive rearing programs continue to date, despite efforts to reduce the prevalence of disease with repeated administration of the antibiotic erythromycin, culling of eggs based on BKD enzyme-linked immunosorbent assay (BKD ELISA) values, and vaccination with a commercial vaccine that has low efficacy in Pacific salmonids (Rhodes et al., submitted). New data on erythromycin treatment suggests that repeated dosing can have a deleterious effect on gamete viability and other measures of reproductive success (see the first report of Task 10, above). However, until safer and more effective chemotherapeutics are identified and proven efficacious for treating BKD in captive broodstock, or until an effective vaccine becomes available, use of erythromycin is likely to continue.

In general, long-term prophylactic use of antibiotics should be avoided where possible. To help reduce dependency on antibiotic treatment, effective vaccines or other non-antibiotic-based therapeutics need to be developed. A number of experimental vaccines have been formulated and tested, but none has proven effective enough for general use. These have included whole-killed cells (bacterins, (Kaattari and Piganelli, 1997)), heat-treated killed whole cells (to reduce the amount of a putative virulence protein, (Piganelli et al., 1999)), and purified p57 .

Recently, Aqua Health Limited licensed a vaccine (Renogen) for use in Canada and the United States for Atlantic salmon. This vaccine is a heterologous vaccine, consisting of a preparation of the bacterium *Arthrobacter sp. nov.* It is reported to induce a cross-

reacting immune response that offers protection against *R. salmoninarum* infection. The bacterium is administered as a live culture and delivered via intraperitoneal (IP) injection. The vaccine underwent safety testing in chinook salmon pre-smolts in trials carried out at NWFSC/NMFS in 2000. It was also used to vaccinate various chinook salmon stocks (starting with brood year 2000) being reared in captive broodstock programs by the Idaho Department of Fish and Game (IDFG) and the Oregon Department of Fish and Wildlife (ODFW). No undue safety or other adverse effects were noted as a result of the vaccination in any of these trials. However, mortality data collected by these programs as well as our own data (Rhodes et al., submitted), strongly suggest that this vaccine fails to protect chinook salmon from BKD, particularly in the second and third years of culture.

MT239 is an attenuated strain of *R. salmoninarum* that expresses very low levels of a 57 kDa immunodominant surface protein called major soluble antigen or MSA (Bruno 1988, O'Farrell and Strom 1999). MSA has been implicated as a virulence factor in BKD pathogenesis (Brown et al. 1996, O'Farrell et al. 2000, Wood and Kaattari 1996), and one of its effects may include immunosuppression (Turaga et al. 1987). MT239 might stimulate an immune response against other *R. salmoninarum* antigens without immunosuppression. When killed, MT239 was combined with the commercially-available vaccine Renogen, and a minor prophylactic and a significant therapeutic effect was observed (Rhodes et al. submitted). The objectives of the studies carried out under the 2002-2003 work plans were to determine whether these whole cell vaccines conferred protection specifically against *R. salmoninarum* and to characterize infection parameters during the period after vaccination with the combination vaccine.

Materials, Methods, and Description of Study Area

Test of specificity of whole cell vaccines

Approximately 400 juvenile fall chinook salmon (*Oncorhynchus tshawytscha*, George Adams stock) were marked with 134.2 kHz passive integrated transponder (PIT) tags twelve weeks prior to seawater transfer. Nine weeks later, the fish were vaccinated intraperitoneally (ip) with a bivalent vaccine against *Vibrio* (Vibrogen-2, Aqua Health) and held three weeks until seawater transfer to the Manchester Research Station. Fish remained in seawater for eight months and were maintained on Nutrafry dry feed (Moore-Clark) for six days per week at 2% of body weight per day. Within one week of the start of the experiment, all fish were treated twice with a 200 mg l⁻¹ formalin bath to remove ectoparasites. Before the start of the experiment, kidney samples were collected from 21 fish and were analyzed for *R. salmoninarum* antigens by enzyme-linked immunosorbent assay (BKD ELISA). The values averaged 0.7155 and ranged from 0.1965 to 3.239, indicating that this population of fish was already infected with *R. salmoninarum*. Using fish already infected with BKD allows further evaluation of the therapeutic utility of the vaccines.

The experimental design used a prime-boost strategy for vaccination. A diagram outlining the vaccine treatments and schedule for vaccination and challenge are outlined

in Figure 1, below. Approximately 100 of the fish were treated in each of the vaccine groups. Inocula consisted of *R. salmoninarum* strain MT239 (1×10^7 cells per fish) and Renogen ($\sim 5 \times 10^4$ CFU per fish), using a phosphate-buffered saline (PBS) vehicle in a total volume of 0.1 ml per fish by intraperitoneal injection. Fourteen days after the booster vaccination, an intraperitoneal challenge of either vehicle (0.1% Bacto-peptone + 0.85% NaCl), *Aeromonas salmonicida* strain A450 (4.5×10^6 CFU per fish), or *R. salmoninarum* strain 33209vir (1×10^6 cells per fish) was administered in a total volume of 0.1 ml per fish. Because all fish were PIT-tagged for unique identification, fish from all four vaccine treatment groups for each challenge were commingled in duplicate challenge tanks (5 foot circular tanks).

Fish were maintained on Nutrafry dry feed (Moore-Clark) for six days per week at 2% of body weight per day until mortality occurred, or the end of the experiment at 110 days post-challenge. Data collected included length, weight, gross pathology observations, kidney weight, information for bacterial septicemia (for cause of mortality), and kidney BKD ELISA. The presence of bacterial septicemia was determined by aseptic inoculation from the kidney onto selective KDM agar plates (for *R. salmoninarum*) and tryptic soy agar plates (for *A. salmonicida*). Survival data was censored using two factors: the growth of the challenge organism on respective agar medium and the value of the kidney BKD ELISA.

Evaluate/assess effect of combinatorial vaccine on BKD infection

Previous work under the 2001-2002 work plan presented evidence for a therapeutic effect of the combined Renogen-killed MT239 whole cell vaccine (Rhodes et al., submitted). The effect of the combined vaccine on infection severity was monitored using BKD ELISA and a quantitative fluorescent antibody technique, QFAT (Cvitanich 1994) and potential host responses by chemokine expression in anterior kidney in a naturally-occurring infection. This study consisted of two parallel experiments with identical vaccination protocols. One sub-experiment monitored mortality while fish in the second sub-experiment were sampled monthly for analysis.

Approximately 250 juvenile fall chinook salmon (*Oncorhynchus tshawytscha*, George Adams stock) representing four crosses were reared from the swim-up stage for approximately one month at the isolation laboratory at the Montlake research facility, then transferred to Manchester and held in 4 foot circular tanks supplied with well water for six months. During this seven-month period, fry were assayed for the presence of *R. salmoninarum* by reverse transcription-PCR (RT-PCR, (Rhodes et al. 1998)) at three time points (10-11 fry per assay per cross). One cross consistently displayed prevalences $\geq 40\%$ positive for *R. salmoninarum*. BKD ELISA values for 20 smolts from this cross were elevated (mean = 0.6472; standard deviation = 0.8832; minimum value = 0.091; maximum value 3.1165), and 18 of the 20 fish were positive for *R. salmoninarum* by QFAT. This cross was subsequently combined for this study. A preliminary effort to PIT tag these salt-water adapted fish resulted in high mortalities (up to 3% mortality per day), a possible result of the combination of BKD and stress associated with tagging. As a result, PIT tagging was not attempted and vaccine treatment groups in the experiment

were maintained in replicate tanks, rather than commingled as in the previous experiment. Four months prior to the start of the experiment, all fish were immersion vaccinated with a bivalent *Vibrio* vaccine (Vibrogen-2, Aqua Health). Because a potentially confounding tail erosion condition was observed, all fish were treated with oxytetracycline (100 mg per kg body weight per day) for ten days by feeding, ending three days before the start of the experiment. All fish were treated three times with a 200 mg 10⁻¹ formalin bath to remove ectoparasites.

The experimental plan consisted of vaccination (with a prime-boost strategy) with either vehicle (PBS) or the combined vaccine (Renogen at ~ 1.4 x 10⁴ CFU per fish; killed MT239 at 5 x 10⁶ cells per fish) in a total volume of 0.1 ml. All fish were maintained in 5 foot circular tanks on Nutrafry dry feed (Moore-Clark) for six days per week at 2% of body weight per day until collected for analysis (sampling sub-experiment), until mortality occurred (both sub-experiments) or until the end of the experiment. An initial sample of 20 fish was collected, and in the sampling experiment, 10 fish per vaccine treatment were collected at monthly intervals, up to six months. Data and samples collected included length, weight, gross pathology observations, anterior kidney for RNA analysis, and posterior kidney for QFAT and BKD ELISA.

Results and Discussion

Test of specificity of whole cell vaccines

The purpose of this experiment was to determine whether the whole cell vaccines could protect by a specific response against *R. salmoninarum* or by stimulation of non-specific immune responses. Because the fish were already infected at the swim-up stage (presumably by infection *in ovo*), mortalities were anticipated and observed, in the mock-challenged groups (Figure 2). Although the Renogen-vaccinated group exhibited the highest survival in the mock-challenged groups, there was no significant difference among the four treatment groups (logrank test, $p = 0.3306$, $df = 3$).

Among fish challenged ip with *R. salmoninarum*, the group vaccinated with Renogen + MT239 exhibited a slight increase in survival in the latter half of the experimental period (Figure 3), but there was no significant difference among the vaccination groups (logrank test, $p = 0.2595$, $n = 3$).

Among the *A. salmonicida*-challenged fish, significant mortalities occurred up to three weeks post-challenge (Figure 4), but challenge-specific mortality halted in all groups by 49 days after challenge. There was no significant difference in survival among the *A. salmonicida*-challenged groups (logrank test, $p = 0.8621$, $n = 3$).

For the mortalities from the mock-challenged and *R. salmoninarum*-challenged fish, BKD ELISA values were high (Figure 5), indicating the cause of death was BKD. In contrast, BKD ELISA values for *A. salmonicida*-challenged mortalities varied widely. When these values were plotted against the day of mortality, a clear pattern of increasing

BKD ELISA value with time emerged (Figure 6). When the results for bacterial septicemia were examined, nearly all of the mortalities that occurred in the *A. salmonicida*-challenged groups beyond 40 days post-challenge had high BKD ELISA values and no bacterial growth on the TSA plates, suggesting these later mortalities were a result of BKD, not furunculosis.

BKD ELISA values of survivors in all groups were low, indicating these fish had low grade or no infections (data not shown).

Evaluate/assess effect of combinatorial vaccine on BKD infection

Prior experiments testing whole cell vaccines relied on survival as a measure of efficacy, and other data (e.g., BKD ELISA) were collected from mortalities or the survivors at the end of the experiment. Previous work showed that survivors in the combination vaccine-treated group exhibited reduced BKD ELISA values, suggesting that these fish had cleared or were clearing the infection. This experiment was designed to evaluate and assess changes in infection that may occur in fish vaccinated with the combined vaccine.

As described in the materials and methods section, two sub-experiments were performed in parallel. One sub-experiment was sampled monthly (10 fish per vaccine treatment group), while the other experiment was not sampled to provide an un-manipulated comparison. Because the fish were not PIT tagged (see Materials and Methods for explanation), triplicate tanks were used for each treatment.

Cumulative survival in both sub-experiments was not significantly different between the mock-vaccinated and combined vaccine treatment groups. Figure 7 shows the cumulative survival (triplicates tanks combined) in the sampled sub-experiment, with the 95% confidence interval for the mock-vaccinated group. Although survival among the combined treatment fish was higher, it was not statistically significant (logrank test, $p = 0.0766$, $df = 1$).

Because the experiment was only recently completed, analysis of sample for BKD ELISA and QFAT are still underway. Analysis for chemokines in the anterior kidney will require efforts to obtain chemokine sequences from chinook salmon prior to performing analyses.

These experiments are extensions of observations from the FY01-02 work period, where a significant therapeutic effect was observed, and a minor prophylactic effect of a combined whole cell vaccine of Renogen and killed MT239. Renogen is a commercially available live vaccine consisting of an *Arthrobacter* spp. with antigenic similarity to *R. salmoninarum* (Griffiths et al. 1998), and MT239 is an attenuated strain of *R. salmoninarum* (Bruno 1988, O'Farrell et al. 2000).

The first study in the current work period addressed the specificity of the protection afforded by the whole cell vaccines. After vaccination with the individual or combined vaccines, we challenged with either *R. salmoninarum* or with a Gram-negative pathogen

that causes the acute disease furunculosis, *A. salmonicida*. If a vaccine elicits immunity through a specific immune response, then protection is anticipated against one pathogen but not against an unrelated pathogen. In this study, no difference in survival was observed among the vaccine treatment groups in the *A. salmonicida*-challenged fish. But there was also no significant protection against *R. salmoninarum* challenge or no significant therapeutic effect in the mock-challenged fish, observations that are not consistent with previous work.

There are several differences between the experimental conditions of the two studies that may explain the disparate outcomes. Although both experiments were performed using the same cohort of fish, the previous study was performed on fish six months younger than those of the current study. Furthermore, the previous study occurred during a period of declining water temperatures, while water temperatures were increasing during the current study. Age and temperature can have effects on immunologic responsiveness (Bly and Clem 1992, Lillehaug et al. 1993, Steine et al. 2001). If these factors explain the differential results, the utility of the combined cell vaccine may be limited to the period immediately after seawater transfer. In Atlantic salmon, this period is a time of increasing total serum IgM (Melingen et al. 1995), which may reflect a developing immune competence. Atlantic salmon also display higher levels of antigen-specific antibodies if vaccinated before smoltification (Melingen et al. 1995, Steine et al. 2001), suggesting that the combined cell vaccine should also be tested in pre-smolts.

The second study was designed to characterize how the combined cell vaccine was reducing mortality in already-infected fish. Here, two parallel sub-experiments were performed, one to be sampled on a regular basis and one to track survival, without the disturbance of collecting fish for sampling. In the sampled sub-experiment, the pattern of cumulative survival was similar to that observed in the previous study (Rhodes et al. submitted), but survival between the mock-vaccinated and combined cell vaccine fish was not statistically significant. One difference between this study and the previous work is the treatment of fish with oxytetracycline prior to the start of this study. Although fish for both studies had been vaccinated against *Vibrio* during the freshwater phase, evidence of a *Vibrio*-associated tail erosion was observed in smolts for this study. To reduce the confounding effect of an acute, fatal infection, oxytetracycline was administered orally for eleven days immediately prior to the start of this study. While oxytetracycline was not anticipated to affect the BKD infection, it could have had an effect. Oxytetracycline has shown some efficacy in reducing vertical transmission of *R. salmoninarum* in coho salmon (Brown et al. 1990), and it has been reported to reduce BKD prevalences among farmed chinook and coho salmon (J. Cvitanich, pers. comm.). Alternatively, the acute tail erosion condition could have affected immune responsiveness to the vaccine. Because the survival pattern for the combined cell vaccine was similar to previous observations, the combined cell vaccine may be efficacious under a variety of disease and treatment conditions.

Data Management Activities

Data are collected by NOAA and PSMFC researchers onto preformatted data sheets or directly into personal computers. Data are entered and summarized on personal computers operated by researchers using programs such as Excel, Filemaker Pro, and GraphPad Prism (for complete list of methods see the Materials and Methods section of this report). All data are checked for quality and accuracy before and after analysis. Analytical processes are described in the text of the annual report under Materials and Methods. Data analyses are reported in the Results section of the annual report.

Summary and Conclusions

Several whole cell vaccines were tested for specificity of protection and therapeutic value. The results suggest that the combined whole cell vaccine of Renogen with killed *R. salmoninarum* strain MT239 may be effective during the period immediately after seawater transfer, but not in yearling seawater-adapted chinook salmon. Whether the immune response was specific against *R. salmoninarum* could not be determined, but work to understand better the application and mechanisms of the effects of the combined cell vaccine will be continued. It is clear that there is no 'silver bullet' against BKD. Control of BKD is likely to require an integrated disease management plan, utilizing three components, namely broodstock segregation, antibiotics, and vaccination. Broodstock segregation has demonstrated success, but there are concerns about genetic impacts of longer-term reliance on that strategy. Antibiotics require prudent and judicious application to avoid development of drug-resistant strains, and efficacious vaccines have been difficult to identify. Vaccine results incorporated with antibiotic treatment will be used to work toward an integrated disease management plan to help to reduce the cycle of BKD transmission in the captive stocks to increase survival safely.

Assessment of whole cell vaccines was performed to extend the previous observations of therapeutic efficacy against natural bacterial kidney disease (BKD) infections. No specific protection against experimental challenge by *Renibacterium salmoninarum* (causative agent of BKD) or *Aeromonas salmonicida* (causative agent of furunculosis) was observed with a commercial vaccine (Renogen), a killed attenuated strain of *R. salmoninarum* (MT239) or a combined vaccine consisting of Renogen and killed MT239. Fish naturally infected with BKD were immunized with the combined vaccine and the course of infection was followed with monthly tissue samples. Vaccinated fish displayed higher survival, a pattern previously observed, but the differences were not statistically significant. A comparison of these results with previous studies suggests that the combined cell vaccine may have therapeutic efficacy during a limited period after seawater transfer.

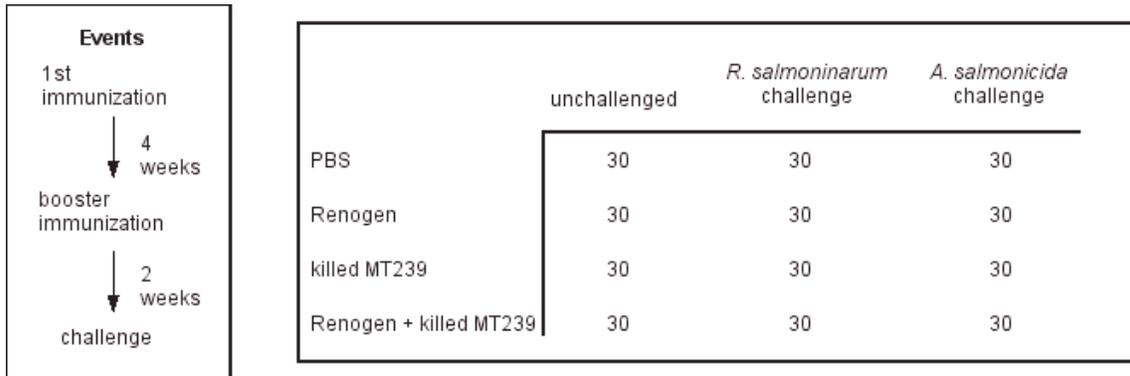


Figure 1. Schedule of treatments and experimental design for experiment to test specificity of whole cell vaccines.

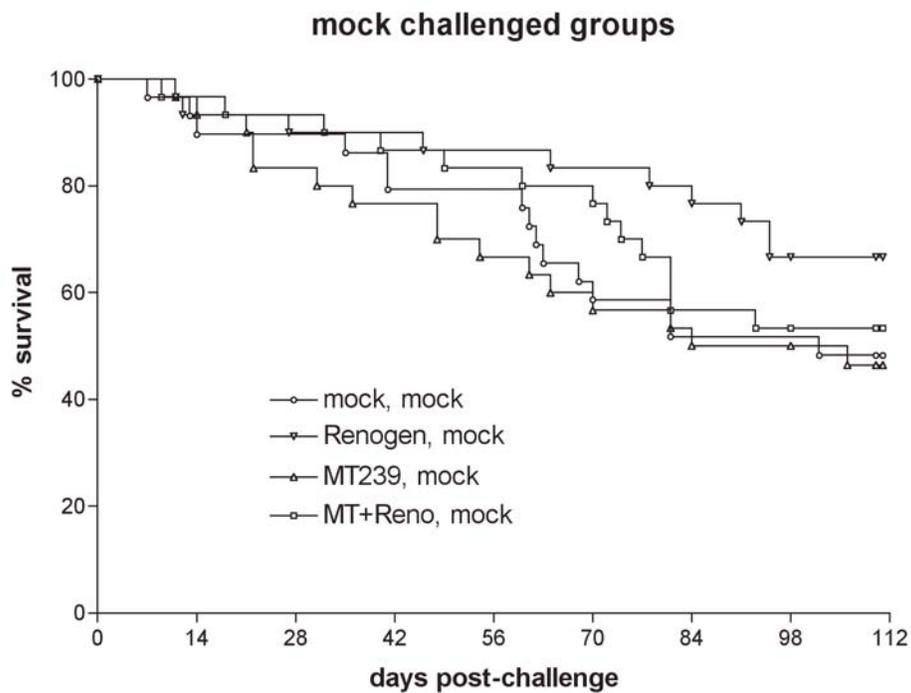


Figure 2. Survival curves of mock-challenged fish that were mock vaccinated (mock, mock) or vaccinated with Renogen (Renogen, mock), killed MT239 (MT239, mock), or combined Renogen and killed MT239 (MT+Reno, mock). X axis is the number of days after challenge, Y axis is the percent survival.

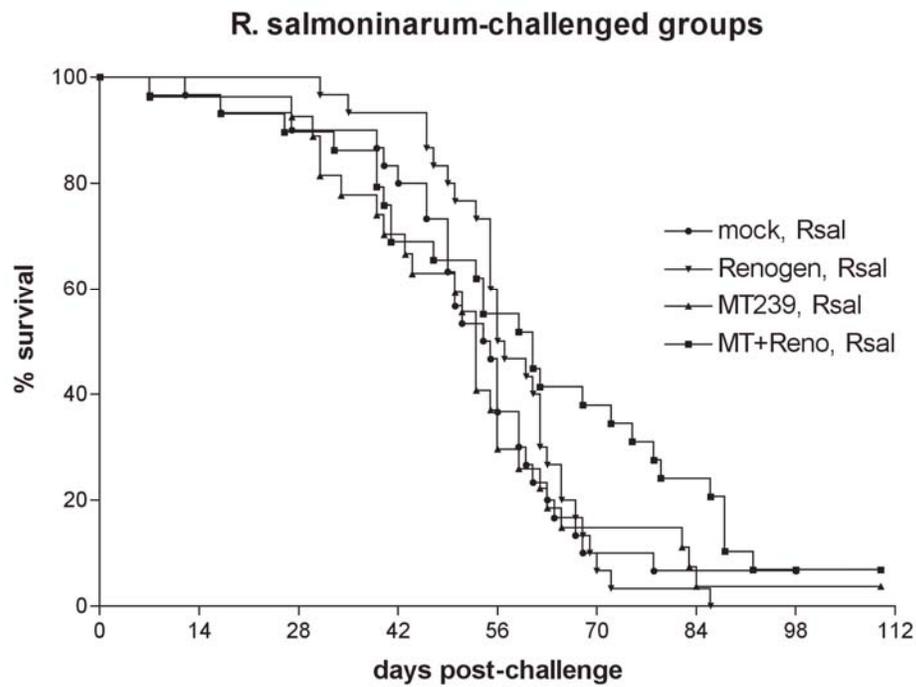


Figure 3. Survival curves of *R. salmoninarum*-challenged fish that were mock vaccinated (mock, mock) or vaccinated with Renogen (Renogen, Rsal), killed MT239 (MT239, Rsal), or combined Renogen and killed MT239 (MT+Reno, Rsal). X axis is the number of days after challenge, Y axis is the percent survival.

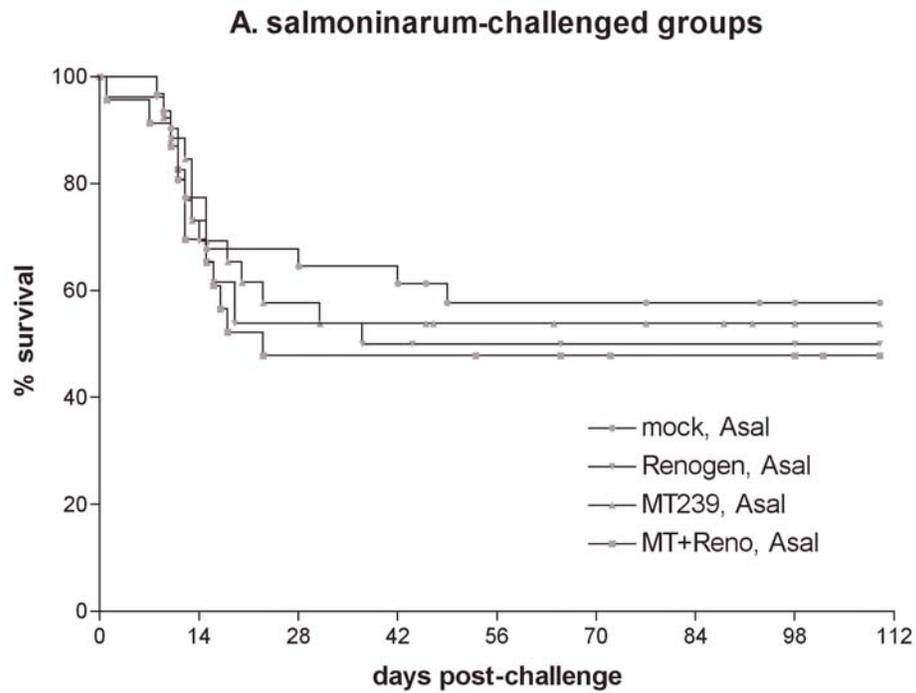


Figure 4. Survival curves of *A. salmonicida*-challenged fish that were mock vaccinated (mock, Asal) or vaccinated with Renogen (Renogen, Asal), killed MT239 (MT239, Asal), or combined Renogen and killed MT239 (MT+Reno, Asal). X axis is the number of days after challenge, Y axis is the percent survival.

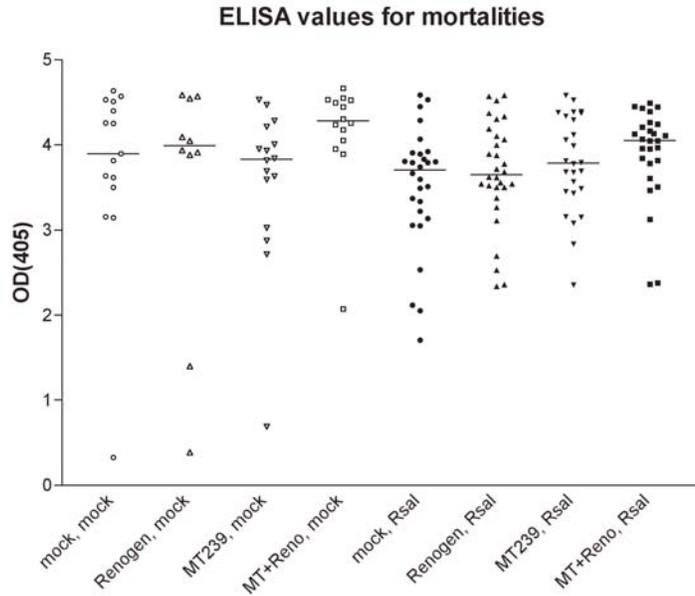


Figure 5. BKD ELISA values for mortalities among mock- or *R. salmoninarum*-challenged fish. The horizontal bars mark the median.

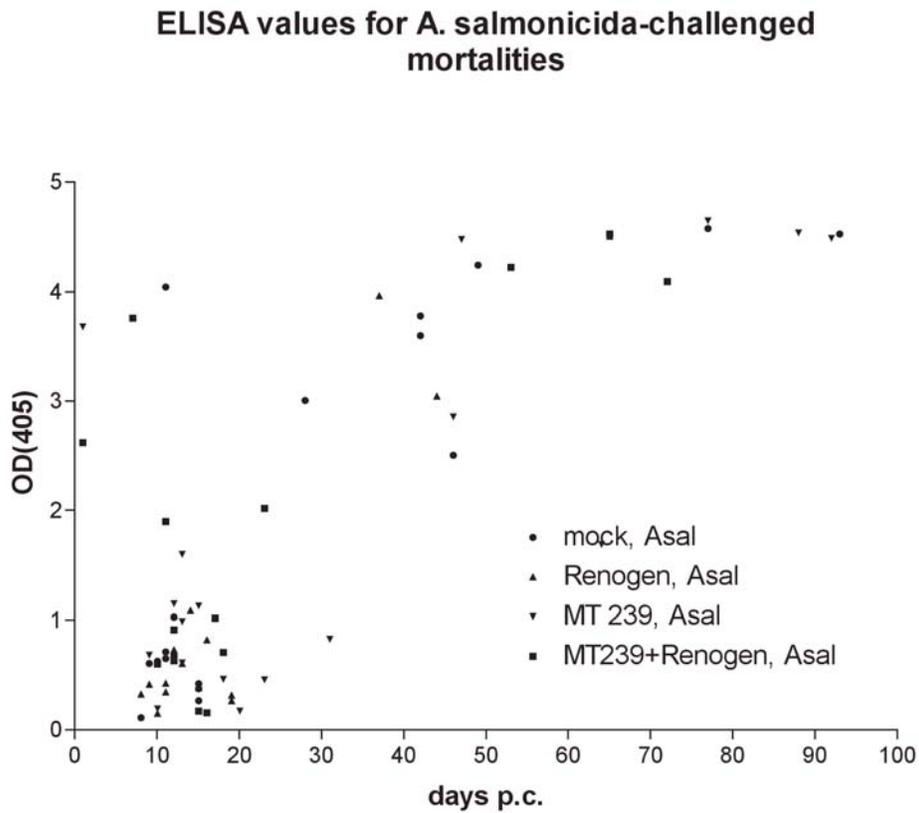


Figure 6. BKD ELISA values for mortalities among *A. salmoninarum*-challenged fish vs the day post-challenge of mortality.

Survival in the Sampled Sub-experiment

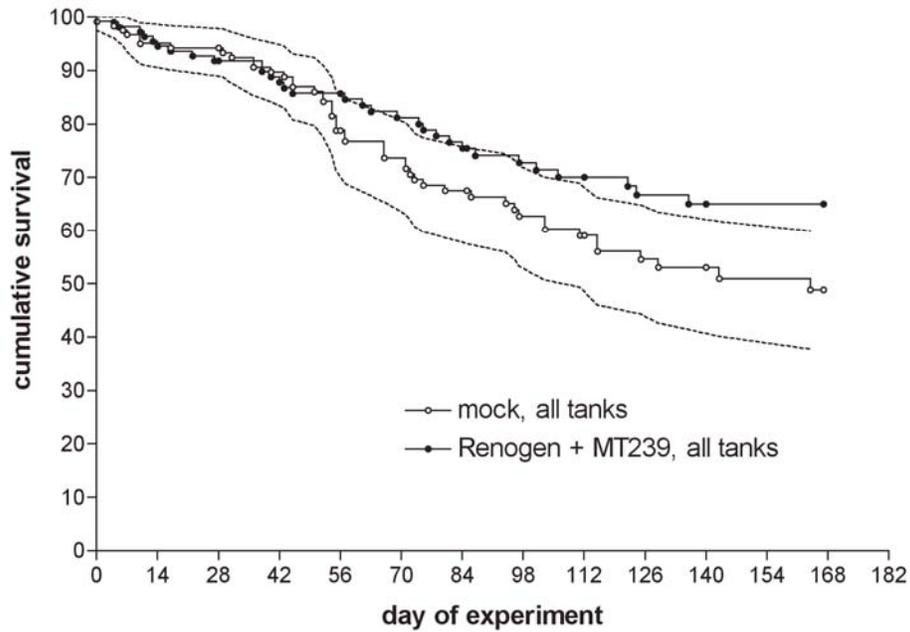


Figure 7. Survival curves fish that were mock vaccinated (mock, all tanks) or vaccinated with combined Renogen and killed MT239 (Renogen+MT239, all tanks) for the second study. The 95% confidence interval for the mock-vaccinated fish is shown as dotted lines. Fish were naturally infected with *R. salmoninarum* prior to the start of the experiment. Samples of ten fish per vaccination group were sampled every 28 days; sampled fish were censored from the survival curve. X axis is the number of days after challenge, Y axis is the cumulative survival.

References

- Bly, J.E., and L.W. Clem. 1992. Temperature and teleost immune functions. *Fish Shell. Immunol.* 2:159-171.
- Brown, L.L., L.J. Albright, and T.P.T. Evelyn. 1990. Control of vertical transmission of *Renibacterium salmoninarum* by injection of antibiotics into maturing female coho salmon *Oncorhynchus kisutch*. *Dis Aquat Organ* 9(2): 127-131.
- Brown, L.L., G.K. Iwama, and T.P.T. Evelyn. 1996. The effect of early exposure of coho salmon (*Oncorhynchus kisutch*) eggs to the p57 protein of *Renibacterium salmoninarum* on the development of immunity to the pathogen. *Fish Shell. Immunol* 6(3):149-165.
- Bruno, D.W. 1988. The relationship between auto-agglutination, cell surface hydrophobicity and virulence of the fish pathogen *Renibacterium salmoninarum*. *FEMS Microbiol. Lett.* 51:135-140.
- Cvitanich, J.D. 1994. Improvements in the direct fluorescent antibody technique for the detection, identification, and quantification of *Renibacterium salmoninarum* in salmonid kidney smears. *J. Aquat. Anim. Health* 6(1):1-12.
- Griffiths, S.G., K.J. Melville, and K. Salenius. 1998. Reduction of *Renibacterium salmoninarum* culture activity in Atlantic salmon following vaccination with avirulent strains. *Fish Shell. Immunol.* 8:607-619.
- Kaattari, S.L., and J.D. Piganelli. 1997. Immunization with bacterial antigens: bacterial kidney disease. *Dev Biol Stand* 90:145-152.
- Lillehaug, A., A. Ramstad, K. Baekken, and L. J. Reitan. 1993. Protective immunity in Atlantic salmon (*Salmo salar* L.) vaccinated at different water temperatures. *Fish Shell. Immunol.* 3:143-156.
- Melinger, G.O., F. Nilsen, and H.I. Wergeland. 1995. The serum antibody levels in Atlantic salmon (*Salmo salar* L.) after vaccination with *Vibrio salmonicida* at different times during the smolting and early post-smolt period. *Fish Shell. Immunol.* 5:223-235.
- Melinger, G.O., S.O. Stefansson, A. Berg, and H.I. Wergeland. 1995. Changes in serum protein and IgM concentration during smolting and early post-smolt period in vaccinated and unvaccinated Atlantic salmon (*Salmo salar* L.). *Fish Shell. Immunol.* 5:211-221.
- O'Farrell, C.L., D.G. Elliott, and M.L. Landolt. 2000. Mortality and kidney histopathology of chinook salmon *Oncorhynchus tshawytscha* exposed to virulent and attenuated *Renibacterium salmoninarum* strains. *Dis. Aquat. Organ.* 43(3):199-209.
- O'Farrell, C.L., and M.S. Strom. 1999. Differential expression of the virulence-associated protein p57 and characterization of its duplicated gene *msa* in virulent and attenuated strains of *Renibacterium salmoninarum*. *Dis Aquat Organ* 38(2):115-123.
- Piganelli, J.D., G.D. Wiens, J.A. Zhang, J.M. Christensen, and S.L. Kaattari. 1999. Evaluation of a whole cell, p57- vaccine against *Renibacterium salmoninarum*. *Dis Aquat Organ* 36(1):37-44.

- Rhodes, L.D., W.B. Nilsson, and M.S. Strom. 1998. Sensitive detection of *Renibacterium salmoninarum* in whole fry, blood, and other tissues of pacific salmon by reverse transcription-polymerase chain reaction. *Mol Mar Biol Biotechnol* 7(4): 70-9.
- Rhodes, L.D., C.K. Rathbone, S.C. Corbett, L.W. Harrell and M.S. Strom (submitted). Efficacy of cellular vaccines and genetic adjuvants against bacterial kidney disease in chinook salmon (*Oncorhynchus tshawytscha*). Included as Appendix A to this report.
- Schiewe, M.H., T.A. Flagg, and B.A. Berejikian. 1997. The use of captive broodstocks for gene conservation of salmon in the western United States. *Bull. Natl. Res. Inst. Aquacult. Suppl* 3:29-34.
- Steine, N.O., G.O. Melingen, and H.I. Wergeland. 2001. Antibodies against *Vibrio salmonicida* lipopolysaccharide (LPS) and whole bacteria in sera from Atlantic salmon (*Salmo salar* L.) vaccinated during the smolting and early post-smolt period. *Fish Shell. Immunol.* 11(1):39-52.
- Turaga, P., G. Wiens, and S. Kaattari. 1987\). Bacterial kidney disease: the potential role of soluble protein antigen(s). *J. Fish Biol.* 31(suppl. A):191-194.
- Wood, P.A., and S.L. Kaattari. 1996. Enhanced immunogenicity of *Renibacterium salmoninarum* in chinook salmon after removal of the bacterial cell surface-associated 57 kDa protein. *Dis. Aquat. Org.* 25(1):71-79.

OBJECTIVE 5 - EVALUATE EFFECTS OF INBREEDING AND INBREEDING DEPRESSION

TASK 9. DETERMINE THE EFFECTS OF CONTROLLED INBREEDING ON SURVIVAL, DEVELOPMENT, AGE STRUCTURE, AND OTHER ASPECTS OF THE LIFE HISTORY OF CHINOOK SALMON

By

Jeffrey J. Hard and Kerry A. Naish

Introduction

Many wild salmon populations exist at low abundance. It is not yet known to what extent inbreeding has reduced and continues to impede productivity of these populations, which aspects of the life cycle are affected most, and whether inbreeding can limit the effectiveness of recovery efforts involving hatchery supplementation or captive broodstocks.

Inbreeding depression, a reduction in fitness caused by the mating of close relatives, has for decades been among the most prominent genetic concerns of captive breeding programs involving threatened or endangered species. This concern stems from adverse effects of inbreeding on survival and reproductive capacity that have been well documented in many species of captive bred animals (Ralls 1983), and experimental work has shown a strong link between the degree of inbreeding and fitness loss (Ralls et al. 1988). A recent study (Saccheri 1998) clearly demonstrated that reduced genetic variation associated with inbreeding can contribute directly to extinction of wild populations. Furthermore, evidence is mounting that a past history of inbreeding (e.g., due to historically small population size) does not necessarily buffer a population from subsequent inbreeding depression (Ballou 1997). The consequences of inbreeding in most salmonids are poorly understood; the relevant work has been limited almost completely to non-anadromous fish, especially brook and rainbow trout (e.g., Kincaid 1976a and 1976b, Gjerde et al. 1983, Su et al. 1996). Nevertheless, studies on these species have found adverse effects of close inbreeding on survival and growth (Hard and Hershberger 1995), and a current review of these studies provides evidence that these effects may occur in other anadromous salmonids as well (Wang et al. 2002).

Even if inbreeding depression leads to higher risk of extinction, it is difficult to evaluate this risk relative to other risks, such as catastrophic loss or domestication of animals in captivity, and population fragmentation or local extinction in the wild. This is particularly true in light of recent evidence that inbreeding depression may reduce fitness

sharply at intermediate levels of inbreeding (Frankham 1995) and its extent is likely to vary in different environments (Pray 1994, Reed et al. 2002).

Research on the consequences of inbreeding in anadromous salmonids would be most useful in characterizing the relationship between inbreeding and inbreeding depression, and the environmental sensitivity of inbreeding depression. For captive broodstock programs, this information would help to evaluate the risk of inbreeding depression against other risks (such as the risk of domestication); this in turn would help to formulate guidelines for determining:

- (i) under what population scenarios a captive broodstock or captive rearing program should (and should not) be initiated based on current inbreeding levels,
- (ii) what captive population sizes should be maintained, and for how many generations, and
- (iii) what characteristics of the captive environment are most important to simultaneously reduce risk of inbreeding depression and domestication.

Materials, Methods, and Description of Study Area

Three basic hypotheses are being tested in this ongoing research project:

* H₀₁: Inbreeding depression does not reduce viability or alter life history characteristics of chinook salmon.

H_{a11}: Inbreeding depression reduces viability during early life history but does not affect development rate, age structure, or reproductive capacity.

H_{a12}: Inbreeding depression has effects throughout the life cycle.

* H₀₂: The degree of inbreeding has no predictable effect on inbreeding depression in chinook salmon.

H_{a21}: The relationship between inbreeding and inbreeding depression is linear.

H_{a22}: The relationship between inbreeding and inbreeding depression is nonlinear (threshold effect).

* H₀₃: Inbreeding depression in chinook salmon does not vary between captive (i.e., protective culture throughout life cycle) and hatchery (i.e., protective culture from embryo to smolt) environments.

H_{a31}: Inbreeding depression is greater in a hatchery than in a captive environment.

H_{a32}: Inbreeding depression is greater in a captive than in a hatchery environment.

In 2002-2003, work continued to address these stated hypotheses through genetic analyses of biological data from first-generation adults returning to their site of release and from experimentally inbred and control captively-reared progeny cultured in marine net-pens. Work is ongoing in Puget Sound, Washington at the University of Washington's School of Aquatic and Fishery Sciences Hatchery (UWH), the site of releases of inbred progeny. Between January 2002 and May 2003, there was no captive experimental fish in culture at the Manchester Research Station (MRS), the site of captive rearing in marine net-pens. In May 2003, the 2002-brood chinook salmon were transferred to MRS for marine culture. Essentially, one complete generation of

experimental inbreeding will be complete with the maturation of adult inbred progeny (through age 5) in November 2003.

Results and Discussion

Preliminary data on the effects of one generation of inbreeding, measured on stage-specific survival and growth during early life history, were summarized in a previous report covering the three years of work between 1996 and 1999. The following sections now focus on the analysis of experimentally inbred progeny, and summarize work on the as-yet incomplete return of first-year (F_2) inbred fall chinook salmon progeny returning to UWH from smolt releases in 1998 and 1999. Results are described, and some preliminary analyses are discussed. A comprehensive summary of the entire project will be provided in a final report by June 2004.

To date, the project has:

- Collected fall chinook salmon broodstock from adults returning to Grovers Creek Hatchery (Puget Sound, Washington) in 1994;
- Established, using a conventional quantitative genetic breeding design (Falconer and MacKay 1996), an experimental population at Grovers Creek Hatchery structured of 96 full-sib families nested within 30 half-sib families;
- In 1995 released to sea 257,093 of these fish from Grovers Creek Hatchery, each identified with full-sib family-specific coded-wire tags;
- Cultured about 500 2-, 3-, and 4-year-old fish marked individually with Passive Integrated Transponder (PIT) tags from the same cohort to maturity in marine net-pens at MRS;
- Spawned over 600 1994-brood adults returning from the 1995 releases or maturing in the marine net-pens between 1996 and 1999;
- Established first-generation inbred lines from matings of 1994-brood parents at UWH. The experimentally inbred lines correspond to a minimal increment in inbreeding (randomly mated control), a moderate increment in inbreeding (half-sib parents, corresponding to an approximate increase in inbreeding, ΔF , of 12.5%), and a substantial increment in inbreeding (full-sib parents, corresponding to an approximate increase in inbreeding, ΔF , of 25%);
- Released to sea from UWH 10,654 1997-brood CWT smolts, composing a total of 28 families in six experimental groups;
- Established at MRS in marine net-pens 3,618 1997-brood PIT-tagged smolts, composing a total of 28 families in six experimental groups;
- Released to sea from UWH 85,111 1998-brood CWT smolts, composing a total of 70 families in four experimental groups;
- Established at MRS in marine net-pens 2,088 1998-brood PIT-tagged smolts, composing a total of 70 families in four experimental groups; and
- Collected biological and coded-wire tag information from approximately 1,600 adults returning to UWH between 1999 and 2002 (total return exceeding 4,000). Most of these tags have been decoded. In these years, few matings were

constructed from these fish because too few experimental females were available for a full mating design. However, six matings of control and first-generation inbred fish were created at UWH in fall 2002 for an experiment to raise non-inbred and inbred fish to maturity to evaluate reproductive behavior and mating success in these two groups. Another 120 matings of UWH stock chinook salmon were also created at UWH in fall 2002, which will provide an opportunity to initiate inbreeding in an independent population as well as a means of estimating the genetic basis of phenotypic variation.

Developmental asymmetry

One prediction of evolutionary theory is that inbreeding, by reducing overall individual heterozygosity, will tend to promote developmental instability. To test this idea, experimental fish were sampled to evaluate variation in bilateral asymmetry, which is thought to reflect minor, random disruptions in an organism's anatomical development. Analyses were completed of 755 juvenile 1998-brood fish from the three experimental groups (control, full-sib inbred, half-sib inbred) to detect evidence for effect of inbreeding on bilateral asymmetry. The three groups of Grovers Creek Hatchery stock chinook salmon in the inbreeding experiment showed significant ($P < 0.05$) differences in levels of fluctuating asymmetry (FA) for eight traits. Two FA indices were computed (Palmer 1994). FA1 was measured as the mean absolute value of the difference between left and right side measurements, A_{ij} , on each fish i for each trait j :

$$FA1_j = \text{Mean } |A_{ij}|.$$

FA5 was measured as

$$FA5_j = \sqrt{\sum (A_{ij})^2 / N_j} \text{ (where } N_j \text{ is the number of fish measured for that trait).}$$

The fish also showed variation in directional asymmetry (DA). For a trait, DA is the raw, signed difference between left and right side measurements, A_{ij} :

$$DA_j = \text{Mean } (A_{ij}).$$

The bilateral traits measured included pectoral fin ray number, pelvic fin ray number, mandibular pore number, branchiostegal number, raker numbers on the first upper and lower gill arches, and raker numbers on the second upper and lower gill arches.

Across groups, correlation matrices indicated that the counts for many, but not all, of the traits were independent. After adjustments for multiple testing using the Bonferroni correction, significant correlations ($P < 0.05$) remained between pelvic and pectoral fin ray number, between lower and upper gill raker number, and between mandibular pores and most of the other bilateral traits, possibly reflecting directional asymmetry. Many trait counts showed significant ($P < 0.05$) departures from normality, and these patterns generally exhibited evidence of negative skewness (long left tail) and leptokurtosis (peaked distribution). These patterns indicate asymmetry and strong modality in the trait counts.

Fish length showed no evidence of detectable correlations with any of the indices of asymmetry. Length was not significantly correlated with AI, FA1, or FA5 (all $P > 0.37$, $r^2 < 0.01$). Traits showing the most variation among samples were pectoral fin ray number, first lower gill arch raker number, and second upper gill arch raker number. For

each of these traits, the groups showed significant ($P < 0.01$) differences in FA and DA; over all traits, the groups also differed significantly (Wilks' lambda = 0.91, $P < 0.0001$).

Among the three groups of experimentally inbred fish (progeny of full siblings, progeny of half siblings, and progeny of unrelated individuals), there were highly significant effects of group for fluctuating asymmetry (FA1 and FA5 indices) and directional asymmetry (DA) (FA1: $F = 9.055$, 2 and 752 df, $P < 0.001$; FA5: $F = 9.556$, 2 and 752 df, $P < 0.001$; DA: $F = 5.615$, 2 and 752 df, $P = 0.004$) (Figure 1). Fish from the unrelated group showed the highest levels of FA1 across all traits; the differences between both the full- and half-sib groups and the unrelated group were responsible for the significant group effect (Bonferroni adjusted $P = 0.001$). This pattern was similar for the FA5 index of fluctuating asymmetry; the differences between both the full- and half-sib groups and the unrelated group were highly significant (Bonferroni adjusted $P = 0.001$). By contrast, fish from the unrelated group showed the lowest levels of DA across all traits; the difference between the half sib and unrelated groups was responsible for the significant group effect (Bonferroni adjusted $P = 0.003$).

The pattern of asymmetry was therefore an unexpected one. Fish from the control (unrelated) matings had the highest degree of FA as measured by both indices. These control fish did, however, exhibit lower levels of DA. At present these results are difficult to interpret, but one possibility is that the FA indices are biased by the presence of DA (Graham et al. 1998), which may account at least in part for these contrasting patterns. Another possibility is that the indices of FA are simply not very sensitive indicators of developmental instability.

In addition to the significant ($P < 0.05$) effects of group on FA1, FA5, and DA (Table 1), nested ANOVA (families nested within groups) indicated highly significant ($P < 0.001$) variation among families in these indices (Table 2). Multivariate tests confirmed the presence of these effects (Group: Wilk's $\lambda = 0.973$, $F = 2.118$, 9 and 1679 df, $P = 0.025$; Family: Wilk's $\lambda = 0.460$, $F = 3.459$, 177 and 2069 df, $P < 0.001$). Estimated broad-sense heritabilities (\pm SE) were 0.60 ± 0.10 for FA1, 0.73 ± 0.10 for FA5, and 0.42 ± 0.09 for DA. These analyses indicate that genetic and/or common environmental variation among families contributed substantially to expressed variation in asymmetry. This is in sharp contrast with the findings of Bryden and Heath (2000). Because of the family structure inherent in a full-sib design, however, the narrow-sense heritabilities for these traits could not be estimated. They could be substantially lower than the broad-sense estimates.

Marine survival

In a previous report, analyses evaluating inbreeding on survival rate during early life history in the hatchery were summarized. Although some significant effects of inbreeding on survival rate were found for the 1998-brood half-sib group, more comprehensive analyses involving ANOVA failed to reveal significant effects of inbreeding among all groups. This is attributed primarily to low power reflecting an insufficient number of families within each group. Retrospective power analyses showed that the greatest power among three separate nested ANOVAs was approximately 10%,

indicating that many more families within each group would be required to detect a significant effect of inbreeding under this pattern of variation. There was also no apparent linear relation between inbreeding depression in survival rate and the level of inbreeding, and no apparent linear relation between variance of the survival rate and the level of inbreeding at any developmental stage.

Ultimately, however, inbreeding depression must be evaluated over the entire life cycle. The most appropriate measure of inbreeding depression is a comparison of lifetime reproductive success between related and unrelated individuals. This test requires an estimate of lifetime fitness, which is not yet available for the fish in this study, as it requires estimates of fecundity and stage-specific survival for all stages over an entire cohort. Nevertheless, inspection of marine survival rates does provide a glimpse of the pattern of fitness differences between inbred and non-inbred individuals.

In 1998 and 1999, 1997- and 1998-brood F2 CWT chinook salmon were released from UWH. Fish from these releases were recovered as they returned to UWH in autumn 1999-2001. In 1999 and 2000, only a few one- and two-year-old males were recovered. In 2001, a total of 2,090 fish returned to the rack between 21 September and 14 November. Of these fish, 1,523 were CWT (235 males and 1,288 females). These fish represented 11 release groups (excluding jacks two years old or younger). In 2002, a total of 344 female and 725 male adults returned to the hatchery pond between 19 September and 25 November. The females averaged (± 1 S.D.) 770.5 ± 79.4 mm fork length and 5.950 ± 1.977 kg round weight; the males averaged 648.3 ± 163.1 mm and 3.440 ± 2.302 kg. Mean body depths were 183.7 ± 25.1 mm for females and 156.6 ± 75.1 mm for males. Mean weight of the egg mass was 1390.84 ± 402.78 g. In a sample of 295 of the coded-wire tagged fish considered large enough to be part of the inbreeding study, 20 were identified as study fish (31 were UWH stock controls).

Each of the 1997-brood study releases was small. These fish were progeny of 3-year-old females, which occur in low frequency in the Grovers Creek Hatchery population, and were in poor condition at time of release from UWH due to an *Aeromonas salmonicida* epizootic. No 1997-brood study fish had been recovered at the UWH rack or in fisheries by 2002, according to the PSMFC coded-wire tag database and UWH records. The 1998-brood releases of study fish, which were progeny of 4-year-old females returning to Grovers Creek Hatchery, were composed of more fish in good overall condition. Based on analysis of the coded-wire tag return data through 2002 (i.e., through age 4 recoveries), the return of adults to the UWH pond from the full-sib group was less than one quarter that of the half-sib and unrelated groups, which had similar return rates. The return rate for the Grovers Creek Hatchery stock study fish to the UWH pond ranged from 0.09% for the full-sib group to 0.35% for both the half-sib group and unrelated control group, while the return rate was 0.90% for UWH stock control fish. Thus, there is evidence for substantial differences in survival, as measured by return rate, among the four groups of fish (Figure 2). The differences were highly significant (Likelihood-ratio test: $G = 1147.53$, 3 df, $P < 0.001$), but much of this difference was due to the higher returns of the endemic UWH stock fish. Excluding that group, the differences in return rates among the three Grovers Creek Hatchery stock fish were still highly significant (G

= 62.62, 2 df, $P < 0.001$), and primarily reflected the lower returns of the full-sib group (0.09%) relative to the half-sib and unrelated groups. Collectively, these preliminary data through age 4 imply that free-ranging Grovers Creek Hatchery stock study fish introduced to UWH had lower survival rates to return than did the local UWH stock control fish, but that inbreeding in progeny of full-sib study fish within the Grovers Creek Hatchery stock further reduced survival substantially.

Although Grovers Creek Hatchery stock study fish survived to return at lower rates than locally adapted UWH stock fish, the patterns of estimated survival within this study were generally consistent with inbreeding depression. Progeny of fish that were full siblings (approximate increment in F of 0.25) survived to return at much lower rates than did progeny of fish that were half siblings ($F \sim 0.125$) or unrelated individuals ($F \sim 0$). Nevertheless, these results must be considered preliminary; returns are not yet complete and not all tags have been recovered from fisheries. Analysis of these data should be complete by June 2004.

Growth and size to adulthood

Comparison of size of adult male and female fish at age illustrates some interesting patterns in growth. The analyses outlined here focus on length data from adults, as these data had the largest sample sizes. In terms of body length, the progeny of Grovers Creek Hatchery full sibs, which had the lowest survival rates, were typically as large as the largest fish returning within an age class, including the UWH stock fish (Figure 3). A factorial ANOVA of the untransformed length data from 1998-brood adults returning in 2001 and 2002 (representing three- and four-year-old adults, respectively) indicated a modest but highly significant variation among the four groups, with the difference among groups explaining about 6% of the total variance (Table 3).

Post-hoc multiple comparisons tests indicate that differences between the UWH control group and the half-sib inbred group were significant ($P < 0.05$) and differences between the UWH control group and the inbred control group were highly significant ($P < 0.01$), after adjustment for multiple testing by Bonferroni correction. Among the Grovers Creek Hatchery stock fish from the inbreeding groups only, the fish did not differ significantly in size (Table 3; $F = 2.48$, 2 and 80 df, $P < 0.10$). For the Grovers Creek Hatchery stock fish, the group effect explained only about 5.7% of the total variance. The general trend for this subset of the data, however, was that adults from the full-sib group were largest on average, with adults either from the half-sib group (2002, age-4 fish) or the control group (2001, age-3 fish) to be smaller (Figure 4). However, the sample sizes for this analysis are small, and the associated statistical power is therefore low.

These results suggest that Grovers Creek Hatchery stock study fish generally grew at lower rates while at sea than did UWH stock fish. Among the inbreeding groups alone, no clear differences in growth were detectable. However, the general pattern of growth was opposite that expected if inbreeding depression reduced growth. It is possible that this pattern reflects a situation in which survival and rapid growth to adulthood are strongly linked to inbreeding level, but this hypothesis is speculative. A comprehensive

analysis of these and other size data must await the return of five-year-old adults in fall 2003.

Composition of 2002-brood fish

A total of 32 adult males were mated to 120 females from the UWH stock between 9 October and 25 November 2002 to create 120 full-sib families in a hierarchical breeding design. These adults were selected from throughout the return of broodstock (Figure 5). In addition, six males were mated to six females from the Grovers Creek Hatchery stock during this period to create six full-sib families: three non-inbred and three first-generation inbred. The primary purpose of the UWH crosses was to establish an independent population sufficiently large to evaluate inbreeding depression adequately, in effect, to replicate the work already under way with the Grovers Creek Hatchery population. The purpose of the current Grovers Creek Hatchery crosses was to provide inbred and non-inbred adults to evaluate reproductive performance, something not yet possible to assess experimentally.

To explore the feasibility of determining effects of inbreeding rates higher than those possible from sexual reproduction within a generation, in 2002 an attempt was made to make two types of presumptive genetic gynogens from the 2002-brood UWH chinook salmon. Gynogenesis is a form of asexual reproduction (parthenogenesis) in which development in an egg is activated by sperm without fusion of the haploid egg and sperm nuclei to form a diploid zygote. Gynogens can be created by initiating egg development with inactivated sperm (e.g., through UV irradiation) and interrupting cell mitosis or meiosis. The first type of gynogen is mitotic, formed from the recombination of two haploid daughter cells before the first mitotic division. Mitotic gynogens are completely inbred ($F = 1.0$), but the survival rate of these fish is typically very low. Indeed, for this study survival was only a fraction of 1% to eyeing. The second type of gynogen is meiotic, formed from retention of the second polar body during meiosis. The rate of inbreeding in meiotic gynogens depends directly on the recombination rate (generally it is in the range of 25-75%), and their survival can be considerably higher (from generally about 20% to perhaps as high as 70% of control progeny, in some cases). However, survival of these fish in these trials was also too low for effective evaluation. It is planned to repeat these trials in 2003 with the information learned about creating them in the hatchery.

At ponding of the UWH fry in winter 2003, there were sufficient numbers of fish for evaluation in 106 full-sib families sired by 32 fathers. In March and April, 30 fish were sampled from each of the full-sib families for measurement of paired lengths and weights and estimation of genetic parameters underlying these traits. The total number of fish measured was 3,180; mean fork length (± 1 S.D.) of these under-yearlings was 67.6 ± 5.0 mm and mean wet weight was 3.7 ± 0.9 g. After accounting for variation among parents in spawning date through ANCOVA, the estimated heritabilities (\pm approximately 1 S.E.) for length and weight were 0.772 ± 0.072 and 0.880 ± 0.074 , respectively; the genetic correlation between these traits was 0.918 ± 0.017 . These results indicate strong genetic control of juvenile size at this stage involving common genes influencing length and

weight. The parameter estimates are similar to those obtained for related traits from the 1994-brood Grovers Creek Hatchery population measured at an equivalent developmental stage (centroid size, a multivariate analog of length: 0.913 ± 0.017 ; weight: 0.995 ± 0.058 ; genetic correlation: 0.943 ± 0.033 ; Hard et al. 1999).

On May 20, 4,000 2002-brood UWH and Grovers Creek Hatchery stock PIT-tagged chinook salmon (mean weight, approximately 8.5 g) were transferred by tank truck to MRS. Beginning on May 23, releases were made from UWH into Portage Bay of 106,000 2002-brood UWH stock coded-wire-tagged fish in 106 families of 1,000 fish (mean weight has not yet been computed, but these fish are of similar size to the PIT-tagged groups). The release was completed by June 2.

Work still to be completed in 2003-2004

- (i) Final collection of experimentally first-generation inbred and control (F_2) 1997- and 1998-brood Grovers Creek Hatchery stock chinook salmon returning to UWH from September to November 2003. It is highly unlikely that sufficient Grovers Creek Hatchery stock adults will return to perpetuate experimental lines by creating second-generation inbred (F_3) fish. It is not intended to repeat the mating design implemented with UWH stock adults in 2003, establishing up to 120 full-sib families nested within 30 half-sib families;
- (ii) Culture of 2002-brood Grovers Creek Hatchery and UWH stock juvenile chinook salmon in seawater raceways and/or net-pens. In the case of Grovers Creek Hatchery stock, these fish represent first-generation inbreds to culture to maturity for evaluation of reproductive behavior and success in relatives and non-relatives. The UWH stock fish represent an attempt to initiate close inbreeding and related genetic studies in an independent stock; and
- (iii) Create gynogens in the UWH stock, and if sufficient fish survive early development, evaluate survival and developmental parameters in these experimental fish.

The following information will be collected for five-year old 1998-brood adults maturing in autumn 2003 and returning to UWH: survival, body length and weight, and for any maturing females, egg size and weight or volume of the egg mass (as a proxy for fecundity). For any of their progeny, data will be collected through the smolt release in June 2004 on stage-specific survival, growth and development rate, and meristic and morphometric variation. Smolts will be marked with group-specific coded-wire tags, with most fish released from the hatchery to sea. If resources permit, up to 4,000 (with up to 30 representatives from each full-sib family) will be PIT-tagged for grow-out to adulthood in captivity.

For next year's report, analysis of inbreeding depression after one generation of inbreeding will be complete. This analysis will be based upon the data collected from 1997- and 1998-brood fish up to 5 years old, which is a complete cohort. Evaluations to determine if the three experimental lines differ in stage-specific survival, size, growth, development rate, and meristic and morphometric variation will be complete by that time.

For the analyses, general linear models and analyses of variance in survival and early growth within and among lines will be used to test the hypotheses stated above and evaluate the effects of inbreeding on these characters, following methods described here and by Lynch (1988), Lynch and Walsh (1998), and Hard and Hershberger (1998). If necessary, these tests will be followed with retrospective power analyses identify the magnitudes of observed differences necessary to detect significant inbreeding depression (Cohen 1988). Survival and growth among the three experimental groups will be compared. For each trait, the coefficient of inbreeding depression (Lande and Schamske 1985) will be computed to provide direct comparison of inbred and control groups. Where it is possible to do so from the survival data, the number of lethal equivalents per gamete will be estimated together with the cost in survival associated with one generation of full-sib mating using the methods described by Morton et al. (1956), Ralls et al. (1988), and Kalinowski and Hedrick (1998).

Data Management Activities

Data are collected by NOAA, UW and Frank Orth & Associates researchers onto preformatted data sheets or directly into electronic spreadsheets or text files. Data are entered and summarized on personal computers operated by researchers; primary software used for these procedures includes Microsoft Excel 2000, Word 2000, and TPSDIG for morphometric analysis. Analytical software used includes Systat 10 (statistics), TPSRELW (morphometrics), Mathematica 4.1 (modeling), and a variety of Fortran and Pascal DOS programs for genetic analyses (e.g., Quercus, DFREML, and MTGSAM). All data are checked for quality and accuracy before analysis. Analytical processes are described in the text of the annual report under Materials and Methods. Data analyses are reported in the Results section of the annual report.

Summary and Conclusions

A summary of data on the effects of one generation of inbreeding, measured on stage-specific survival and growth during early life history, in a previous report covering three years of work between 1996 and 1999. A summary of the comprehensive genetic and phenotypic analyses of the biological data collected from the first-generation parents will be provided in the next report. A final report will be submitted when one full generation of experimental inbreeding is implemented with the maturation of adult inbred progeny through age 5, by June 2004. In that report, the relationship between inbreeding and inbreeding depression, and the environmental sensitivity of inbreeding depression in light of the hypotheses presented above, will be characterized.

This report summarizes analyses of the as yet incomplete return of first-year (F2) inbred fall chinook salmon progeny returning to UWH from smolt releases in 1998 and 1999. Incomplete returns of first-generation inbred chinook salmon (through age 4) reveal some interesting patterns of response in survival, developmental asymmetry, and growth in

response to inbreeding. Grovers Creek Hatchery stock study fish have thus far survived to return at lower rates than locally adapted UWH stock fish.

Within the Grovers Creek Hatchery inbreeding study fish, the patterns of estimated survival were generally consistent with inbreeding depression: progeny of fish that were full siblings (approximate increment in F of 0.25) survived to return at much lower rates than did progeny of fish that were half siblings (F ~0.125) or unrelated individuals (F ~0). Progeny of full-and half-siblings showed lower levels of fluctuating asymmetry (a measure of developmental instability) in bilateral characters than did progeny of unrelated parents, but higher levels of directional asymmetry (another measure of deviations from an expected developmental program). In addition, significant variation among families in these metrics was detected, indicating high estimates of broad-sense heritability. Growth at sea of Grovers Creek Hatchery stock study fish was lower than that of UWH stock fish. Among the inbreeding groups alone, no clear differences in growth were detectable. However, the general pattern of growth was opposite that expected if inbreeding depression reduced growth: the highest growth was in progeny of related parents. These results should be considered preliminary.

Table 1. Factorial analyses of variance of indices of fluctuating asymmetry (FA1 and FA5) and directional asymmetry (DA) for 8 bilateral traits measured in three groups of 1998-brood juvenile chinook salmon at UWH in April 1999. Groups were progeny of full siblings, progeny of half-siblings, and progeny of unrelated individuals. See text for interpretation and results of hypothesis testing.

Source	Sum of Squares	df	Mean Square	F-ratio	P
DA	1.01	3	0.338	2.698	0.045
Error	86.76	692	0.125		
FA1	0.80	3	0.267	3.510	0.015
Error	52.72	692	0.076		
FA5	317.10	3	105.699	3.988	0.008
Error	18340.84	692	26.504		

Table 2. Nested analyses of variance of indices of fluctuating asymmetry (FA1 and FA5) and directional asymmetry (DA) for 8 bilateral traits measured in 60 families of 1998-brood juvenile chinook salmon at UWH in April 1999. The families were nested within the three groups described in Table 1. See text for interpretation and results of hypothesis testing.

Source	Sum of Squares	df	Mean Square	F-ratio	P
DA	28.50	59	0.483	3.853	0.000
Error	86.76	692	0.125		
FA1	25.61	59	0.434	5.698	0.000
Error	52.72	692	0.076		
FA5	11214.15	59	190.070	7.171	0.000
Error	18340.83	692	26.504		

Table 3. Factorial analyses of variance of fork length of 1998-brood adult chinook salmon returning to UWH in 2001 (age 3 fish) and 2002 (age 4 fish). Top table (A): ANOVA of three Grovers Creek Hatchery stock groups (full-sib mated, half-sib mated, unrelated control) as well as the UWH stock control. Bottom table (B): ANOVA of Grovers Creek Hatchery stock groups only. See text for interpretation and results of hypothesis testing.

(A)

Source	Sum of Squares	df	Mean Square	F-ratio	P
Sex	244.36	1	244.36	0.039	0.844
Group	132378.91	3	44126.30	7.011	0.000
Sex X Group	43103.97	3	14367.99	2.283	0.079
Error	1963651.76	312	6293.75		

(B)

Source	Sum of Squares	df	Mean Square	F-ratio	P
SEX\$	1423.14	1	1423.142	0.209	0.649
GROUP\$	33857.31	2	16928.655	2.484	0.090
SEX\$*GROUP\$	7240.85	2	3620.425	0.531	0.590
Error	545218.34	80	6815.229		

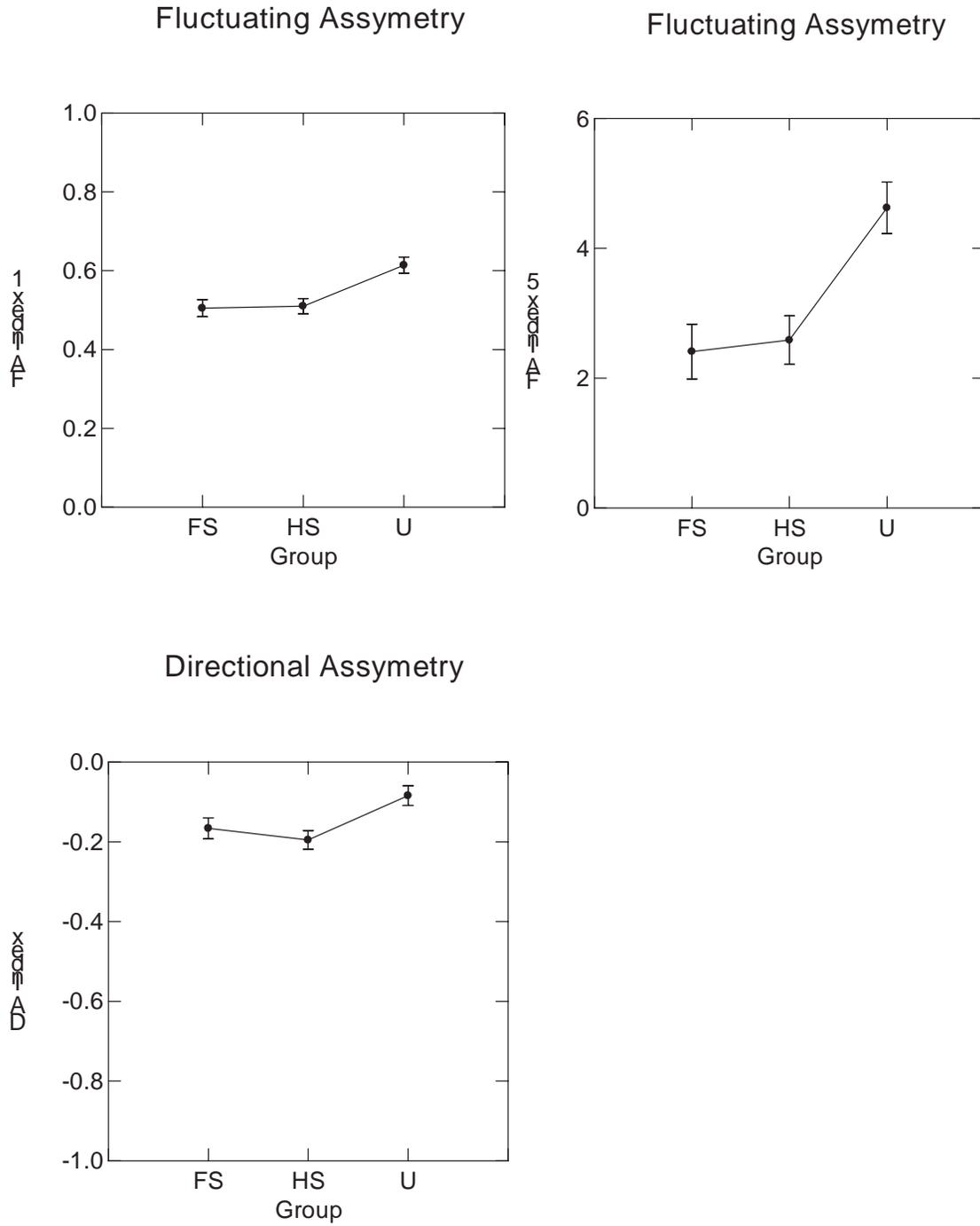


Figure 1. Mean size-corrected indices of fluctuating asymmetry (FA index 1 and FA index 5) and of directional asymmetry (DA) for 8 bilateral traits measured in 1998-brood Grovers Creek Hatchery stock juvenile chinook salmon at UWH in April 1999 (mean FL 87.2 ± 0.3 mm). Groups: FS, progeny of full siblings; HS, progeny of half siblings; U, progeny of unrelated individuals.

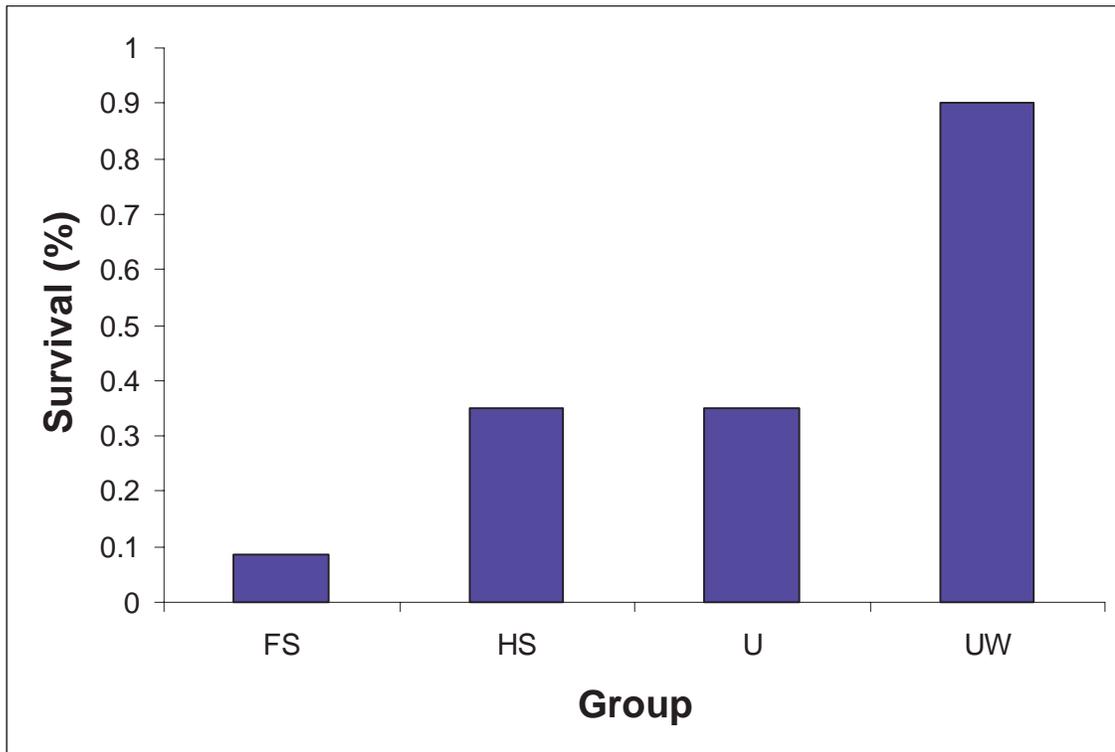


Figure 2. Return rates (%) of 1998-brood Grovers Creek Hatchery stock and UWH stock chinook salmon to UWH through age 4 (1998-2002). Grovers Creek Hatchery stock groups: FS, progeny of full siblings; HS, progeny of half siblings; U, progeny of unrelated individuals. UW, UWH stock unselected control.

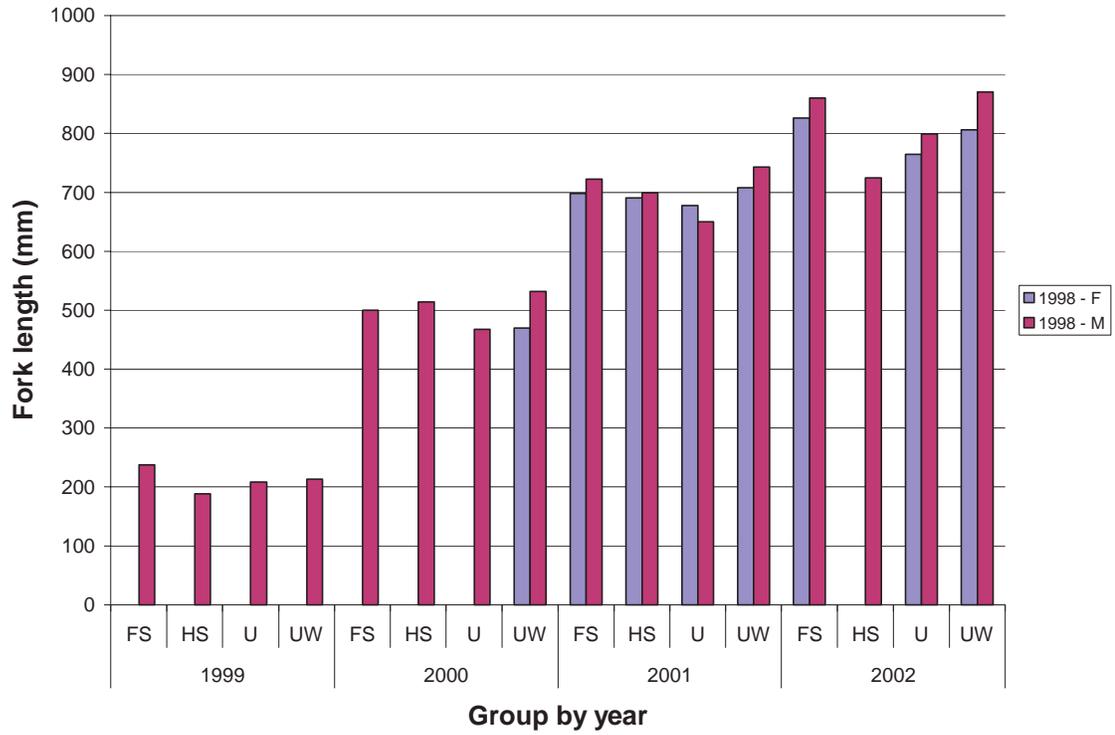


Figure 3. Fork lengths of 1998-brood Grovers Creek Hatchery stock and UWH stock chinook salmon recovered at UWH from 1999-2002. Grovers Creek Hatchery stock groups: FS, progeny of full siblings; HS, progeny of half siblings; U, progeny of unrelated individuals. UW, UWH stock unselected control.

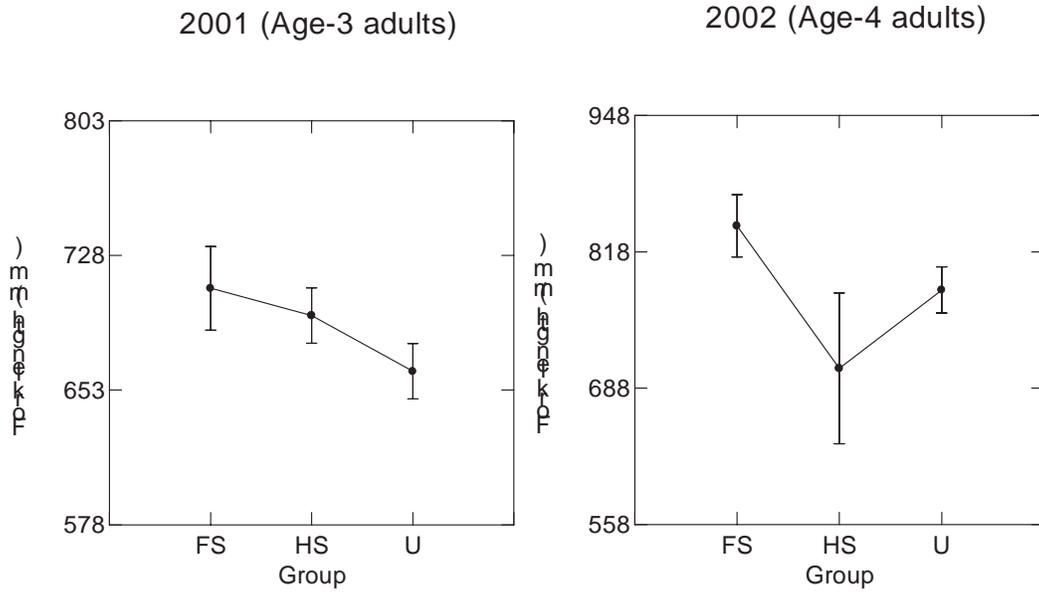


Figure 4. Comparison of adult fork lengths among 3- and 4-year-old 1998-brood adult Grovers Creek Hatchery stock chinook salmon returning to UWH in 2001 and 2002. Data are means \pm 1 S.E. See text for interpretation.

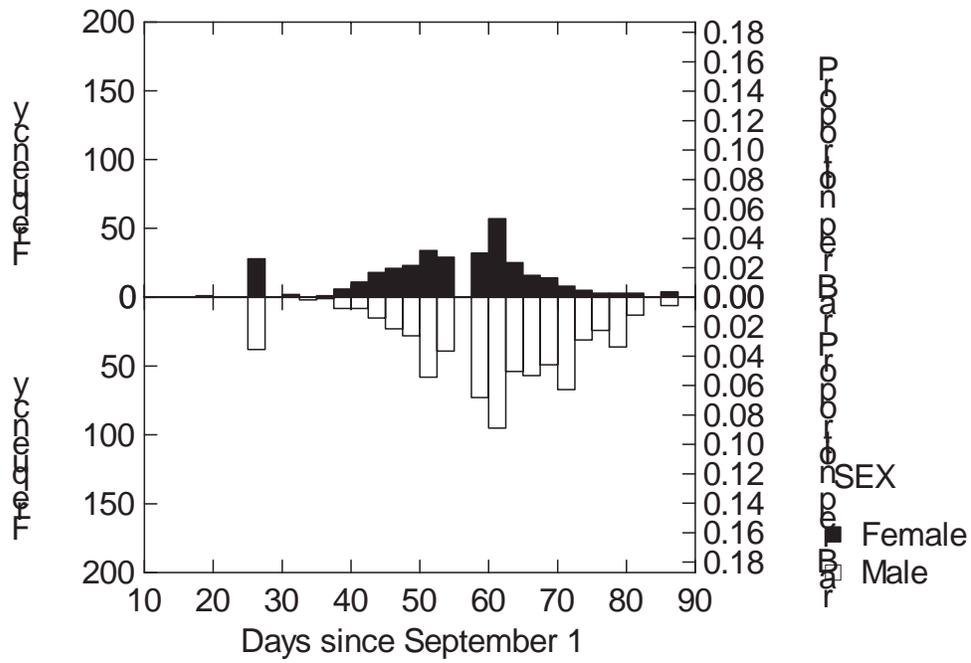


Figure 5. Frequency distribution of spawning dates of adult chinook salmon at UWH, September to November 2002. Broodstock were selected from throughout this distribution (19 September to 25 November) in proportion to their abundance.

References

- Ballou, J.D. 1997. Ancestral inbreeding only minimally affects inbreeding depression in mammalian populations. *J. Hered.* 88:169-178.
- Cohen, J. 1988. *Statistical power analysis for the behavioral sciences*, 2nd ed. Lawrence Erlbaum Associates, Hillsdale, NJ, 567 p.
- Bryden, C.A., and D.D. Heath. 2000. Heritability of fluctuating asymmetry for multiple traits in chinook salmon (*Oncorhynchus tshawytscha*). *Can. J. Fish. Aquat. Sci.* 57:2186-2192.
- Frankham, R. 1995. Inbreeding and extinction: a threshold effect. *Conserv. Biol.* 9:792-799.
- Falconer, D.S., and T.F.C. Mackay. 1996. *Introduction to quantitative genetics*, 4th ed. Addison Wesley Longman Ltd., Harlow, Essex, U.K.
- Gjerde, B., K. Gunnes, and T. Gjedrem. 1983. Effect of inbreeding on survival and growth in rainbow trout. *Aquaculture* 34:327-332.
- Graham, J.H., J.M. Emlen, D.C. Freeman, L.J. Leamy, and J.A. Kieser. 1998. Directional asymmetry and the measurement of developmental instability. *Biol. J. Linn. Soc.* 64:1-16.
- Hard, J.J., and W.K. Hershberger. 1995. Quantitative genetic consequences of captive broodstock programs for anadromous Pacific salmon (*Oncorhynchus* spp.). In T.A. Flagg and C.V.W. Mahnken (editors), *An assessment of the status of captive broodstock technology for Pacific salmon*, p. 2-1 - 2-75. Available Bonneville Power Administration (Project No. 93-56), P. O. Box 3621, Portland, OR 97208.
- Hard, J.J., and W.K. Hershberger. 1998. Research on quantitative genetic consequences of captive broodstock programs for Pacific salmon populations, Part VIII, p. 8-1 to 8-28. Bonneville Power Administration, P. O. Box 3621, Portland, OR 97208-3621. Research on captive broodstock technology for Pacific salmon (Proj. 93-56).
- Hard, J.J., G.A. Winans, and J.C. Richardson. 1999. Phenotypic and genetic architecture of juvenile morphometry in chinook salmon. *J. Hered.* 90:597-606.
- Johnson, O.W., K.A. Neely, and R.S. Waples. 2003. Lopsided fish in the Snake River Basin--fluctuating asymmetry as a way of assessing impact of hatchery supplementation in chinook salmon (*Oncorhynchus tshawytscha*). *Environ. Biol. Fish.* (in press).
- Kalinowski, S.T., and P.W. Hedrick. 1998. An improved method for estimating inbreeding depression in pedigrees. *Zoo Biol.* 17:481-497.
- Kincaid, H.L. 1976a. Effects of inbreeding on rainbow trout populations. *Trans. Am. Fish. Soc.* 105:273-280.
- Kincaid, H.L. 1976b. Inbreeding in rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Board Can.* 33:2420-2426.
- Lande, R., and D.W. Schemske. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* 39:24-40.
- Lynch, M. 1988. Design and analysis of experiments on random drift and inbreeding depression. *Genetics* 120:791-807.

- Lynch, M., and B. Walsh. 1998. Genetics and analysis of quantitative traits. Sinauer Associates, Inc., Sunderland, MA, 980 p.
- Morton, N.E., J.F. Crow, and H.J. Muller. 1956. An estimate of the mutational damage in man from data on consanguineous marriages. *Proceedings of the National Academy of Sciences, USA* 42:855-863.
- Palmer, A.R. 1994. Fluctuating asymmetry analyses: a primer. Pp. 335-364 *in* T.A. Markow, ed. *Developmental instability: its origin and evolutionary implications*. Kluwer Academic Publishers, Dordrecht, the Netherlands.
- Pray, L.A., J.M. Schwartz, C.J. Goodnight, and L. Stevens. 1994. Environmental dependency of inbreeding depression: implications for conservation biology. *Conserv. Biol.* 8:562-568.
- Ralls, K., and J. Ballou. 1983. Extinction: lessons from zoos. *In* C. M. Schonewald-Cox, S. M. Chambers, B. MacBryde and L. Thomas (editors), *Genetics and conservation*, p. 164-184. Benjamin/Cummings, Menlo Park, CA.
- Ralls, K., J. Ballou, and A. Templeton. 1988. Estimates of lethal equivalents and the cost of inbreeding in mammals. *Conserv. Biol.* 2:185-193.
- Reed, D.H., D.A. Briscoe, and R. Frankham. 2002. Inbreeding and extinction: the effect of environmental stress and lineage. *Conserv. Genet.* 3:301-307.
- Saccheri, I., M. Kuussaari, M. Kankare, P. Vikman, W. Fortelius, and I. Hanski. 1998. Inbreeding and extinction in a butterfly metapopulation. *Nature* 392:491-494.
- Su, G.-S., L.-E. Liljedahl, and G.A.E. Gall. 1996. Effects of inbreeding on growth and reproductive traits in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 142:139-148.
- Wang, S., J.J. Hard, and F. Utter. 2002. Salmonid inbreeding: a review. *Rev. Fish Biol.* 11:301-319.

APPENDIX A

OBJECTIVE 3 - IMPROVE IN-CULTURE SURVIVAL OF JUVENILES: PREVENTION AND CONTROL OF DISEASE

FINAL REPORT FOR TASK 10 FROM FY 1999 - 2002. USE OF ANTIBIOTICS AND NEW VACCINES TO REDUCE MORTALITY FROM BACTERIAL KIDNEY DISEASE IN CHINOOK SALMON

By

William T. Fairgrieve, Cyndy L. Masada, William C. McAuley, Mark E. Peterson,
Mark S. Myers, and Mark S. Strom

Introduction

Bacterial kidney disease (BKD) is the major infectious disease affecting the successful culture of salmonids in the Pacific Northwest. In 1993 and 1994 this disease was responsible for catastrophic losses of endangered Redfish Lake sockeye salmon held as captive broodstock. BKD-caused epizootics continue to impact captive rearing programs of both sockeye and chinook salmon (Schiewe et al. 1997).

As of yet there is no vaccine available to completely protect salmon from infections with *Renibacterium salmoninarum*, the causative bacterium of BKD. To date, erythromycin, administered orally through feed or by injection of maturing adults, has been the primary antibiotic used by fish culturists in an attempt to prevent and control *R. salmoninarum* (Elliott et al. 1989). The antibiotic is commonly administered prophylactically to control epizootics of BKD in captive rearing and captive broodstock programs. However, while use of erythromycin usually results in short-term health improvement of infected fish, it fails to completely eliminate the infection and symptoms of disease, and recurrence of disease in treated fish is common.

In addition, results of preliminary studies with Lake Wenatchee sockeye salmon suggest that erythromycin may have a negative effect on gamete viability. Recent experiences with Catherine Creek, Lostine River, and Lemhi River spring chinook salmon have shown currently mandated treatment regimens may elicit fatal toxicity reactions. In part because of these toxic reactions, another macrolide antibiotic, azithromycin, has been tested for the treatment of BKD in captive broodstock salmon.

In experiments carried out from 1996 – 1999 under the BPA Captive Broodstock Research Program, azithromycin was shown to have a strong bactericidal activity against *R. salmoninarum* both *in vitro* and *in vivo*, and is effective in reducing clinical symptoms

of BKD, improving long-term survival through spawning. The effectiveness of the drug probably lies in its ability to concentrate in polymorphonuclear leukocytes, macrophages, and fibrocytes (Peters et al. 1992), all cell types that *R. salmoninarum* is known to invade, and be sequestered from the salmonid immune system (Bandín et al. 1993; Gutenberger et al. 1997).

Until an effective vaccine becomes available, erythromycin and/or azithromycin will continue to be used to control BKD in captive rearing and broodstock programs. It is important that this use follows guidelines designed to minimize the development of resistant pathogen strains. With this in mind, work has been carried out towards development of a more efficacious vaccine (see the report of Rhodes, et al., in this same report). The vaccine results will be incorporated with antibiotic treatment to work toward an integrated disease management plan to reduce the cycle of BKD transmission in the captive stocks to safely increase survival. One important aspect of this type of integrated disease management is to design treatment regimens that avoid antibiotic-induced toxic reactions and reductions in reproductive success having first determined the causes of the reactions and conditions under which they appear.

In Study I the accumulation and clearance of orally-administered erythromycin and azithromycin in juvenile fall chinook salmon (*Oncorhynchus tshawytscha*) was examined; in Study II, it is the effects of repeated oral administration of erythromycin phosphate to chinook salmon. The work on both is detailed in this report.

Study I: A comparative evaluation of accumulation and clearance of orally-administered erythromycin and its derivative, azithromycin, in juvenile fall chinook salmon (*Oncorhynchus tshawytscha*)

For more than 20 years, the macrolide antibiotic erythromycin has been the chemotherapeutant of choice to control bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum* in salmon. Although prophylactic treatment with erythromycin may delay the onset of clinical disease in infected fish, it fails to eliminate the infection completely and symptoms often return after treatment ends. There are no proven vaccines available to prevent BKD in Pacific salmonids, and there is a clear need for alternative chemotherapeutants.

Azithromycin, a new macrolide antibiotic derived from erythromycin, is one candidate. Unlike erythromycin, which is bound to serum protein and largely remains in extracellular spaces, azithromycin concentrates in polymorphonuclear leukocytes, macrophages and fibrocytes (Peters et al. 1992). These are all cell types which *R. salmoninarum* has been shown to invade (Bandín et al. 1993, Gutenberger et al. 1997). Azithromycin has strong *in vitro* bactericidal activity against *R. salmoninarum* (Fryer 1987) and has demonstrated *in vivo* efficacy in experimentally-infected salmonids.

Patterns of accumulation and depletion of orally-administered erythromycin in yearling chinook salmon have been previously evaluated (Moffitt and Schreck 1988). However, there are no published comparative studies on the pharmacokinetics of azithromycin and

erythromycin in fish. The objective of this is to compare the uptake, retention and clearance of orally-administered erythromycin and azithromycin by fall chinook salmon (*Oncorhynchus tshawytscha*) during the first-feeding to smolt stage.

Materials, Methods, and Description of Study Area

Experimental fish and husbandry

In January 1999, unfed fry of fall chinook salmon (initial weight 0.42 g) obtained from the University of Washington research hatchery located at Big Beef Creek were randomly distributed among 14 tanks until each contained 225 fish. The experiment was conducted in 1.83 m diameter (1,250 l) circular polyethylene tanks. Temperature (10°C) and flow (10 l/min) were held constant throughout the experiment. Natural photoperiod was simulated in the laboratory with two levels of incandescent lighting, and adjustments in day length were made daily.

The fish were acclimated to laboratory conditions for 14 days and fed to apparent satiation (*ca.* 6% body weight/day) with a semi-moist starter diet (BioOregon starter diet, BioOregon, Inc., Warrenton, OR). Thereafter, a growth model based on the delta-1 method of Butterbaugh and Willoughby (1967) was used to calculate the daily ration required to produce smolts of *ca.* 16 g by June 15, 1999. Feeding schedules were adjusted according to actual growth and feed conversion data collected during monthly samplings.

Antibiotic administration

Duplicate groups of fall chinook salmon were fed diets medicated with azithromycin (A) or erythromycin (E) either one (AS-1, ES-1), two (AS-2, ES-2), or three times (AS-3, ES-3) over a period of 140 days, beginning two weeks after first feeding (February) and ending at smoltification (June). Dietary drug concentrations and feeding rates were adjusted during each feeding period to deliver 30 mg (active) azithromycin/kg body weight per day for 14 days or 100 mg (active) erythromycin/kg body weight per day for 28 days (Table 1). Unmedicated diets were fed to the control fish throughout the study and to the test groups between antibiotic treatments and during the withdrawal period (141 – 216 days post-ponding).

Commercially available non-sterile erythromycin phosphate (Abbot Laboratories, North Chicago, IL; 74.5% anhydrous potency) and azithromycin dihydrate (Zithromax® 250 mg tablets; Pfizer Inc., New York, NY) were used for the test drugs. Medicated feeds were prepared by dissolving the antibiotics in 100% ethanol then spraying the mixture (25 ml/kg feed) onto a dry commercial salmon diet (Nutri-fry salmon diet, Moore-Clark Canada, Inc., Vancouver, BC). The feed was mixed thoroughly and the ethanol allowed to evaporate at room temperature. Pellets were frozen (–20°C) until used.

Collection and storage of tissues

At the beginning of the feeding trial, before and after each antibiotic treatment, and 41 and 76 days after the final treatment period, fish from each group were sampled to measure growth and whole body antibiotic concentrations. After the fish in each tank were bulk weighed and counted, ten randomly selected fish were sacrificed by immersion in a solution of tricaine methanesulfonate (>200 ppm), blotted on damp paper towels, and placed into individual Whirl-pak® bags. The samples were stored frozen (−20°C).

An additional five fish were collected before each treatment and 41 days after the final treatment period for histological evaluation. Samples of gill, head and trunk kidney, liver, spleen, heart, pyloric caeca, gonad, and brain were fixed in a freshly prepared solution of Davidson's fixative (30 parts 95% methanol/20 parts 40% formalin (paracide F)/10 parts glycerine/10 parts glacial acetic acid/30 parts distilled water). After 24 – 36 h, they were transferred to a solution of 70% (v/v) ethanol in distilled water and stored under refrigeration (5°C) until further processing.

Antibiotic assays for erythromycin and azithromycin

The concentration of erythromycin and azithromycin in fish tissue was determined microbiologically using the bacterium *Micrococcus luteus*, which is sensitive to erythromycin concentrations as low as 0.0025 µg/10 µl of sample (Evelyn et al. 1986a). The method was modified from the agar diffusion method (Difco 1968; pp. 13-15) described by Evelyn and coworkers (1986a, 1986b). The plate base layer (45 ml of Difco antibiotic medium 11 (Difco Laboratories, Detroit, MI) was poured into 150 x 15 mm sterile petri dishes and overlaid with 13.5 ml Difco antibiotic medium 11 inoculated with 0.2 ml of a *Micrococcus luteus* culture (1.0 OD₅₄₀). Individual whole fish were placed in separate Whirl-pak® bags, weighed, then an equivalent volume of phosphate buffer solution (pH 7.2) was added to dilute the samples. The samples were then homogenized with a rubber roller. Onto each inoculated plate, five sterile 7 mm paper disks (Becton Dickinson Microbiology System, Cockeysville, MD) were evenly placed around the plate, and one disk was placed in the center. Aliquots (10 µl) of the diluted homogenates were micropipetted onto the disks. Aliquots of erythromycin or azithromycin reference standards (2.0 µg/ml) were spotted on the center disk of each plate. Plates were incubated at 30°C for 24 hours after which the diameter of the zones of inhibition were measured to the nearest 0.5 mm. Antibiotic concentration in each test disk was calculated from a standard curve (inhibition zone diameter vs. log₁₀ antibiotic concentration in µg/ml) and corrected for dilution to determine equivalent antibiotic concentrations in the whole fish carcass (µg/g wet weight).

Histology

Prior to sectioning, the fixed tissues were routinely dehydrated and processed for embedment using a Shandon Hypercenter XP automated tissue processor, followed by embedment in Polyfin (Triangle Biomedical Sciences, Durham, NC). Tissues were sectioned at a thickness of 4 – 5 µm using a Reichert-Jung Biocut 2030 microtome.

examined under a Nikon Optiphot light microscope. Specimens examined for histopathological lesions in this study were confined to fish tissue samples collected 41 days after the final drug treatments, which represented the most extreme and longest duration of erythromycin and azithromycin treatments possible. Five fish per replicate tank (two replicate tanks per treatment) for a total of ten fish per treatment and 20 control fish were sampled at this time point. All sections were examined by a single examiner with many years of experience in fish histopathology in a “blind” system in which the examiner was not aware of the treatment the fish had been subjected to until all specimens had been examined.

Statistical methods

Effects of the antibiotic treatment regimens on growth were assessed by one-way analysis of variance (followed by Dunnett’s test), comparing mean weights of treated and control groups at the end of the dosing phase of the experiment. The relationship between size (age) at first exposure and accumulation of azithromycin or erythromycin was evaluated by non-linear regression analysis of whole-body antibiotic concentration versus weight. Student’s t-test or analysis of variance was performed on $\log_{10}(1 + \text{concentration})$ transformed data to determine whether antibiotic type or treatment frequency affected accumulation of the drug in the tissues. The kinetics of azithromycin residue clearance from the tissues during the final withdrawal phase of the experiment were described using a one phase exponential decay model obtained by non-linear regression of whole-body antibiotic concentration versus time (GraphPad Prism Statistical Software, GraphPad Software, Inc., San Diego, CA). Half-lives were the quotient 0.6932 over the elimination rate constant. Statistical analysis of the experimental results was performed using JMP® statistical software (SAS Institute Inc., Cary, NC). A level of significance of $p \leq 0.05$ was used in all statistical analyses.

Results and Discussion

Feed acceptability and growth

Diets medicated with erythromycin or azithromycin were well accepted by juvenile fall chinook salmon, and no palatability problems were observed at the dietary concentrations tested (Table I-1). Average weight gain in treated and control fish was similar, except during the final treatment period when a trend toward reduced growth was observed in both antibiotic groups (Figure I-1). Differences in the average weights of untreated (control) fish and those treated with azithromycin were not significant ($p = 0.57$). Compared with the control group, growth was significantly reduced only in the ES-3 group ($p = 0.02$).

Drug accumulation

Azithromycin was concentrated in the tissues to a greater extent than was erythromycin during all feeding periods, at all treatment frequencies (Figure I-2). After a daily oral dosage of 100 mg/kg for 28 days, maximum tissue concentrations of erythromycin

ranged from 0.2% of the daily dosage in first-feeding fry, to about 2.5% and 10% at the midpoint and end of the trial. In contrast, mean tissue concentrations in fish fed azithromycin at a daily oral dosage of 30 mg/kg for 14 days ranged from about 60% of the daily dosage in first-feeding fry, to 115% and 150% as the trial progressed. Observed tissue antibiotic concentrations ranged from 4 – 100-fold higher in groups treated with azithromycin compared with those treated with erythromycin. The greatest differences were noted among groups of first-feeding fry.

A pattern of increasing post-treatment tissue concentration was observed in groups administered azithromycin or erythromycin two (AS-2, ES-2) or three times (AS-3, ES-3), but no significant effect of treatment history was found (Figure I-2). On day 77 (azithromycin) and day 91 (erythromycin), the average concentration of antibiotics in fish that had received a total of one (of two) or two (of three) treatments did not differ significantly (AS-2 vs. AS-3, $p = 0.30$; ES-2 vs. ES-3, $p = 0.15$). Similarly, there were no significant differences in whole-body antibiotic concentrations among groups administered single (AS-1, ES-1) or multiple treatments with either azithromycin ($p = 0.61$) or erythromycin ($p = 0.71$).

Additional analysis showed that the increased tissue concentrations of both antibiotics observed as the trial progressed were related to fish size (or age), rather than to the number of treatments previously administered (Figure I-3). A log-linear relationship ($R^2 = 0.99$) between azithromycin accumulation in the tissues of chinook salmon and weight was observed. After a single 14-day treatment, two-fold increases in whole body concentrations of azithromycin occurred in 4 – 12 g fish, compared with first-feeding fry (0.5 g). In contrast, tissue concentrations of erythromycin increased exponentially ($R^2 = 0.96$) with fish size, and were 10 – 60-fold greater in fish averaging 5 – 14 g than in first-feeding fry.

Drug clearance

Compared with erythromycin, azithromycin was remarkably persistent in the tissues of juvenile chinook salmon. Azithromycin remained detectable in fish tissues for at least five weeks during the first-feeding to smolt stage (Figure I-2) and for 11 weeks following drug withdrawal (Figure I-4). In contrast, erythromycin was cleared from the tissues within three weeks after medicated feed was discontinued. Based on whole body concentrations, the elimination half-life of azithromycin in fish fed single or multiple doses of 30 mg/kg was about 14.7 days (Figure I-4).

Histopathology

No lesions consistent with toxicity were observed in the excretory elements of the trunk kidney of fish from any of the erythromycin or azithromycin treatments or untreated controls; however, incidental findings of single, minor cases of nephrocalcinosis were observed in fish from the following treatments: untreated controls, ES-2, AS-1, and AS-2. No significant lesions were detected in liver, exocrine pancreas, gill, pyloric caeca, upper intestine, heart, head kidney, spleen, and gonad. Female fish from all treatment and control groups displayed oocytes at the late perinucleolus stage of maturation (secondary

oocytes) (Nagahama 1983). Male fish from all groups were primarily at the regressed stage of testicular maturation, composed almost entirely of Type A or primary spermatogonia, with a few fish showing early and late recrudescence (up to the development of spermatids). Males in these later stages of testicular maturation were rare, and were distributed evenly among the treatment and control groups.

Medicated diets were well accepted by juvenile chinook salmon, and no reductions in palatability were observed at concentrations as high as 2,560 mg azithromycin or 8,145 mg erythromycin per kg of feed. In contrast, Schreck and Moffitt (1987) found that yearling chinook salmon smolts offered diets containing 6,700 mg erythromycin thiocyanate per kg consumed fewer pellets than those offered an unmedicated control feed. Acceptability further declined when the dietary antibiotic concentration was increased to 10,000 and 12,000 mg/kg. Reasons for these differences in sensitivity are not known, although it is possible that the thiocyanate form of erythromycin is less palatable than the phosphate form used in the present trial.

Azithromycin was better absorbed and retained in chinook salmon tissues than was erythromycin during all feeding periods. Despite a lower dietary concentration (740 – 2,560 mg/kg vs. 2,710 – 8,145 mg/kg) and reduced treatment duration (14 days vs. 28 days), accumulated tissue concentrations of azithromycin were 100 times higher at the start, 10 – 20 times higher at the midpoint and four times higher at the end of the trial than those in the corresponding erythromycin-treated groups. Numerous studies with animals other than fish also report increased tissue penetration and absorption of azithromycin over erythromycin (Peters et al. 1992).

Quantitative differences in tissue antibiotic concentrations for fish of different size or age have important implications for the design of therapeutic regimens. Bandín et al. (1991) found that *in vitro* MICs for erythromycin against 11 strains of *R. salmoninarum* ranged from 0.62 – 5.47 µg/ml, while 50% of the strains had MICs \geq 2.73 µg/ml. In terms of bactericidal activity, however, erythromycin killed 50% of the strains at a concentration of 5.47 µg/ml and 90% of the strains at a concentration of 21.87 µg/ml. In our study, accumulated concentrations of erythromycin in first-feeding fry (0.19 µg/g) were only 3.5% of the concentration considered bacteriostatic to 90% of the strains tested. Marginally inhibitory concentrations of erythromycin were found in fish averaging 5.6 g (1.84 – 3.18 µg/g), but bactericidal concentrations (9.97 – 11.20 µg/g) were attained only at smoltification, when they weighed 15.6 g.

Comprehensive studies measuring the minimum inhibitory and bactericidal concentrations for azithromycin against *R. salmoninarum* have not been conducted. However, Fryer (1987) found the *in vitro* MIC for azithromycin against four strains of *R. salmoninarum* (type strains ATCC 33209 and 33739; D-6, a USA isolate from coho salmon; and 932, a Norwegian isolate from Atlantic salmon) to be only one-half (0.05 µg/ml) of the *in vitro* inhibitory concentration for erythromycin (0.1 µg/ml). Given these data, results from the present study with azithromycin are especially noteworthy. Concentrations of azithromycin in the whole body tissues of chinook salmon ranged from 18.98 µg/ml in first-feeding fry, to nearly 50 µg/ml in smolts. Azithromycin concentrates

in polymorphonuclear leukocytes, macrophages and fibrocytes (Peters et al. 1992), cell types *R. salmoninarum* is known to invade (Gutenberger et al. 1997). Thus, it seems likely that therapeutically efficacious tissue levels of azithromycin can be attained, even in first-feeding fry.

In this study, erythromycin was eliminated from the tissues of juvenile chinook salmon within three weeks of the last antibiotic feeding. A pattern of erythromycin retention by muscle, kidney, liver, and blood was determined for yearling chinook salmon administered erythromycin thiocyanate in the feed at 100 mg/kg for 21 days (Moffitt and Schreck 1988). Erythromycin was not detectable in the blood or plasma 10 days after treatment, but was present in the kidney and liver after 19 days. By contrast, azithromycin followed a protracted elimination phase: residues of the antibiotic were found in all treated groups 74 days after treatment. The high tissue concentrations and prolonged retention of azithromycin in chinook may increase the effectiveness of the antibiotic against *R. salmoninarum* compared with erythromycin. Additional research to measure the bioavailability of orally-administered azithromycin, concentrations bactericidal to *R. salmoninarum in vivo*, and pharmacokinetics of the antibiotic will be required to establish therapeutic regimens for its use to treat BKD in captive broodstocks of endangered chinook salmon.

Table I-1. Treatment schedule, sizes of fish (g) and dietary concentrations of azithromycin and erythromycin. Dietary antibiotic concentrations were adjusted based on actual feeding rates and feed conversion ratios to provide daily dosages of 30 mg azithromycin or 100 mg erythromycin per kg fish body weight.

Treatment period (days post- ponding)	Treatments	Range of weights (g)	Dietary concentration (mg base/kg)
14 – 27	AS-3	0.5 – 0.6	740
14 – 41	ES-3	0.5 – 1.3	2,710
63 – 76	AS-3, AS-2	3.0 – 5.2	900 – 1,225
63 – 90	ES-3, ES-2	3.0 – 5.6	3,000 – 4,594
113 – 126	AS-3, AS-2, AS-1	11.1 – 13.0	1,890 – 2,560
113 – 140	ES-3, ES-2, ES-1	11.0 – 15.3	6,300 – 8,145

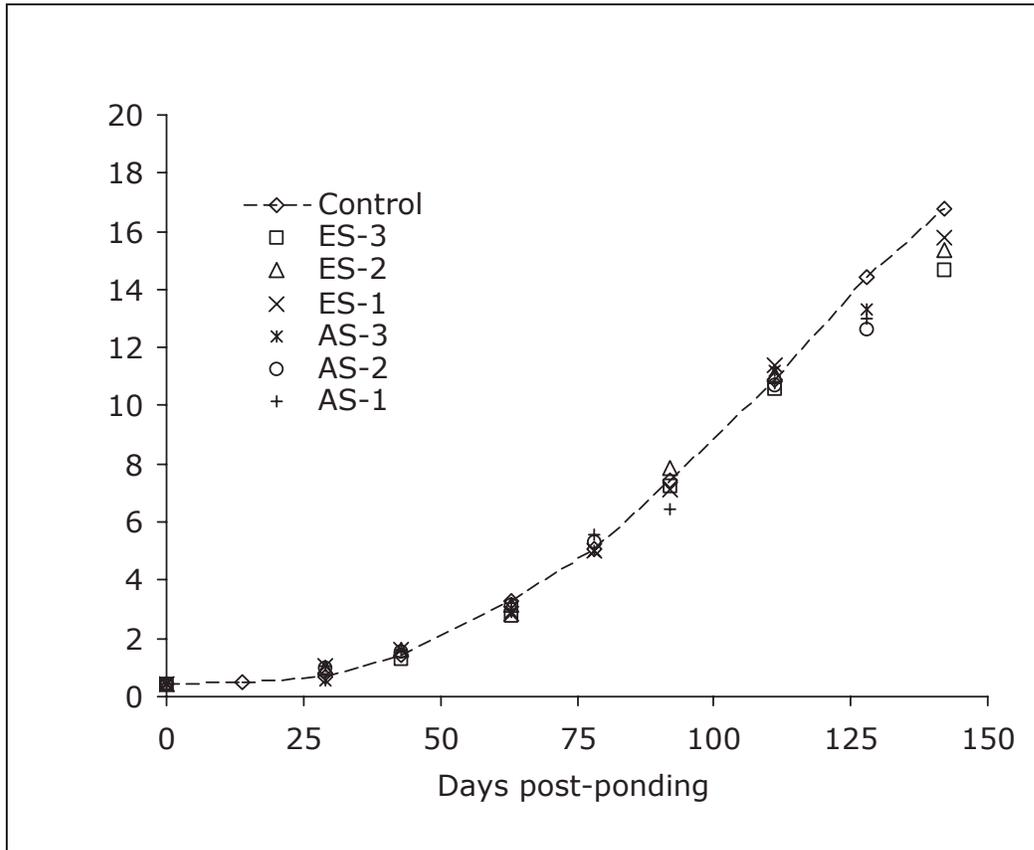


Figure I-1. Growth of juvenile fall chinook salmon fed diets containing either azithromycin (AS) or erythromycin (ES), one (AS-1, ES-1), two (AS-2, ES-2), or three (AS-3, ES-3) times in Study I.

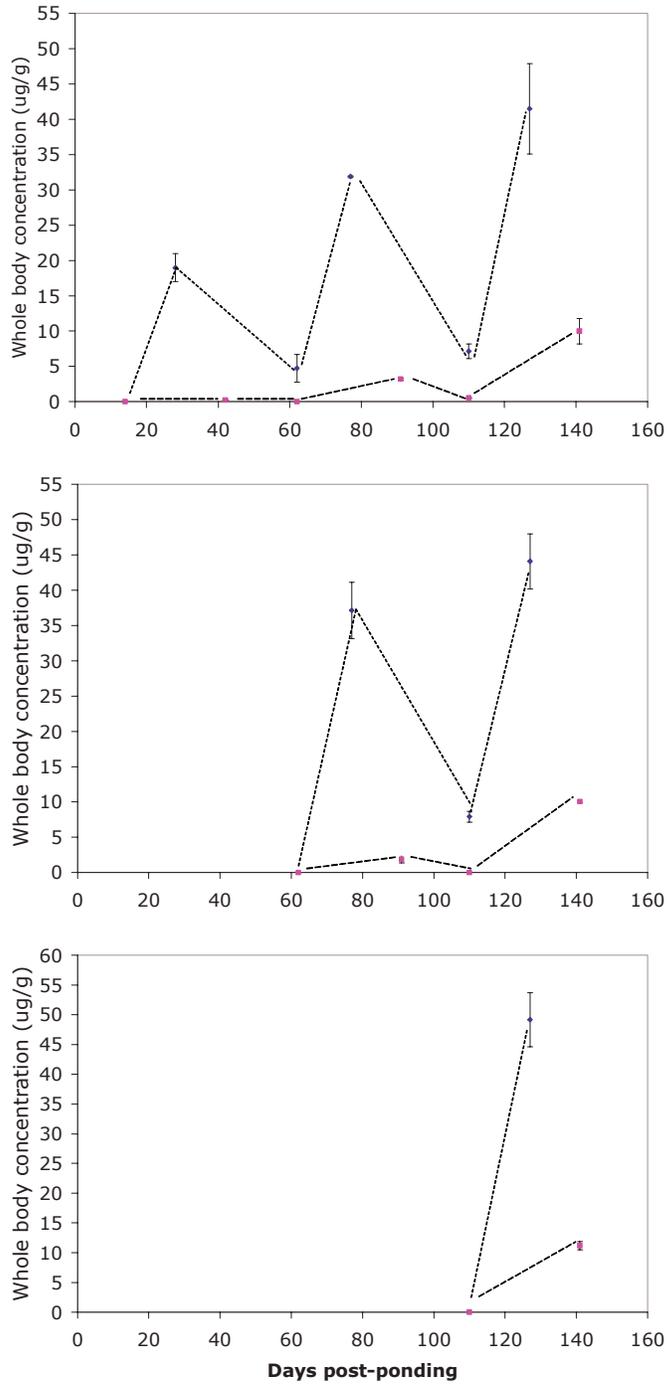


Figure I-2. Effects of three (top), two (middle) or one (bottom) oral dosage(s) with azithromycin (diamonds) or erythromycin (squares) on pre- and post-treatment whole body antibiotic concentrations in juvenile fall chinook salmon. Data are means (\pm SE) of two tanks per treatment.

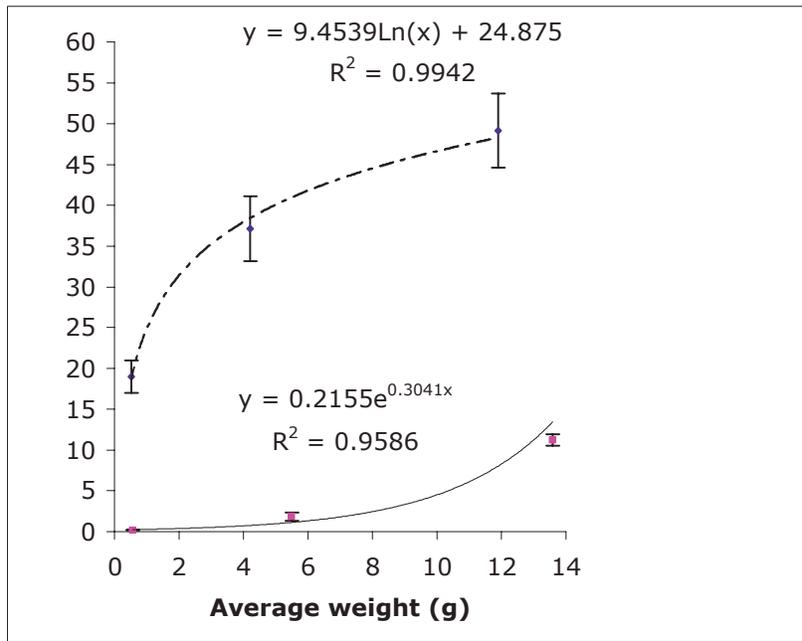


Figure I-3. Relationships between fish weight and accumulation of azithromycin and erythromycin in the tissues of juvenile chinook salmon after a single orally-administered treatment ($n = 2 \pm SE$).

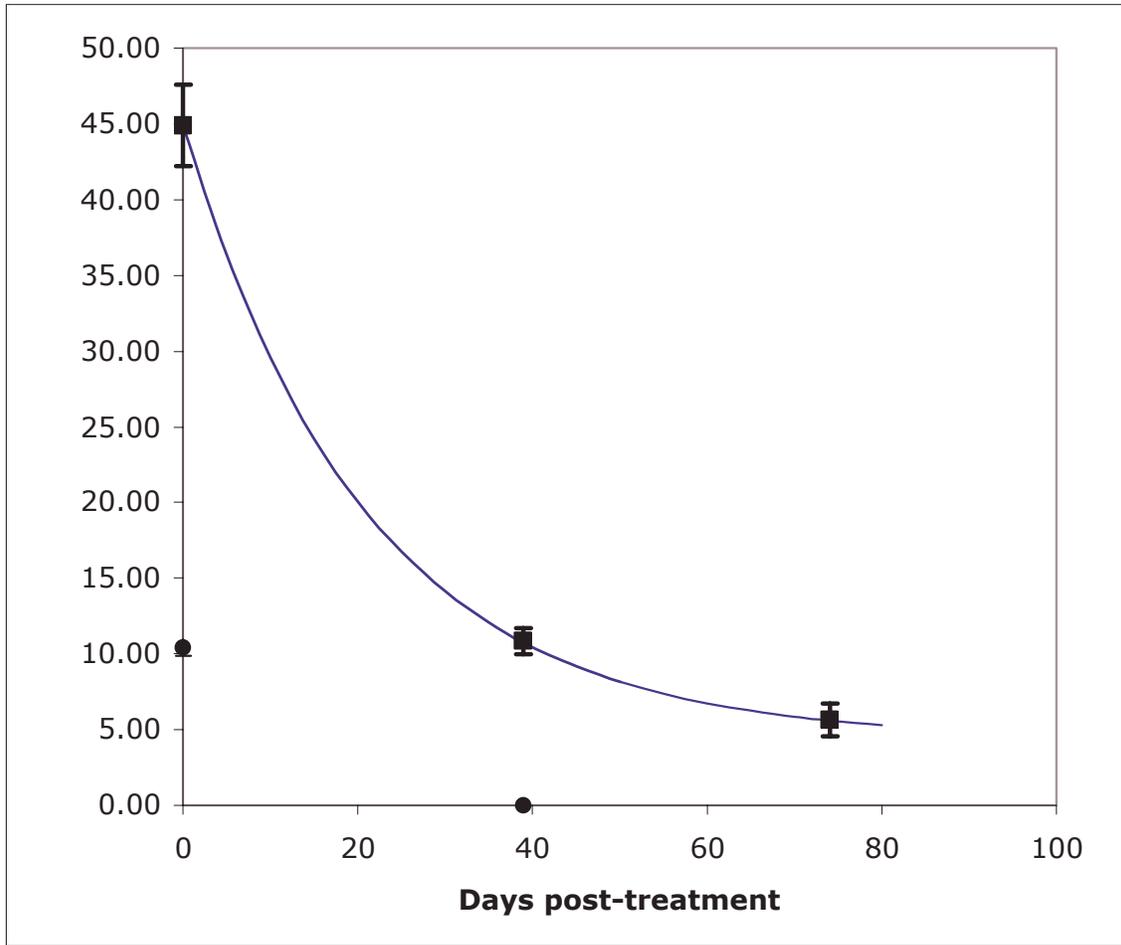


Figure I-4. Mean antibiotic concentrations in tissues of fall chinook salmon after the last dose of azithromycin or erythromycin. The elimination half-life for azithromycin was 14.7 days (n = 6).

Study II: Chronic toxicity following repeated oral administration of erythromycin phosphate to fall chinook salmon (*Oncorhynchus tshawytscha*)

Erythromycin has been used since the 1950s for preventing and treating bacterial kidney disease (BKD) in salmonids (Wolf and Dunbar 1959). It has been administered by injection to maturing adults, used when water hardening eggs to reduce vertical transmission (Groman 1983), and as a feed additive for treating epizootics in pre-smolt salmonids. Prophylactic administration of erythromycin has become increasingly common, particularly in captive broodstock programs involving ESA-listed stocks of sockeye and chinook salmon.

Controlled studies to determine the effects of erythromycin on gamete quality are especially relevant to captive rearing of ESA-listed stocks. Research conducted by Flagg et al. (2000) suggested that orally-administered erythromycin negatively affects gamete viability in sockeye salmon (*Oncorhynchus nerka*). Survival of eyed eggs from sockeye prophylactically treated up to four times per year and spawned during 1994 and 1995 ranged from 40 – 60%. In addition, ovarian inflammation in rainbow trout fed erythromycin thiocyanate has been reported (Piper 1961); however, no studies have been conducted measuring the effects of repeated, long-term exposure to erythromycin on gonad development and egg viability in any salmonid species.

Experiences with Catherine Creek, Lostine River, and Lemhi River spring chinook salmon have shown that repeated treatment with erythromycin may elicit fatal toxicity reactions. Observed effects have included reddened skin in the cranial region and erratic swimming followed by convulsions and death. Extensive liver damage has also been observed. Experience has shown that terminating treatment alleviates the red-head condition. Similar toxic reactions have been reported in rainbow trout (Piper 1961) and juvenile chinook salmon (Moffitt 1998). No data currently exist for chinook salmon during the post-smolt life cycle stage, and it is not known if sensitivity increases with repeated treatments.

The objectives of this study with captive fall chinook salmon broodstock were to: (1) measure the effects of pre- and post-smolt treatments on growth and survival; (2) determine how gonad development, gamete viability, and survival of their progeny through the swim-up stage are affected by long-term prophylactic administration of erythromycin; (3) determine whether acute erythromycin toxicity responses are related to treatment frequency; and (4) document any underlying histopathological changes associated with erythromycin treatment.

Materials, Methods, and Description of Study Area

Experimental fish, husbandry, and antibiotic administration

In June 1999, fall chinook salmon smolts (initial weight 20.5 g) that had been used in the first-feeding fry-to-smolt portion of this series of studies (Study I) were PIT-tagged and

redistributed so a secondary treatment could be applied. Briefly, in Study I, groups of fry were fed an unmedicated control diet, or diets medicated with either erythromycin (E) or its chemical derivative, azithromycin (A), one to three times over a period of five months (treatment codes ES-1, ES-2, ES-3, AS-1, AS-2, and AS-3). At smoltification, the fish were distributed among six 4 m diameter (12 m^3) tanks until each contained 40 fish from the seven pre-smolt treatments (280 fish/tank). Temperature (10°C) and flow (135 l/min) were constant throughout the feeding phase of the experiment.

For Study II, duplicate groups of fall chinook salmon were fed diets medicated with erythromycin either two (E-2) or four times (E-4) per year for two years, beginning in early September 1999 and ending in late June 2001, 78 days before spawning was initiated in mid-September 2001. Dietary drug concentrations and feeding rates were adjusted during each feeding period to deliver 100 mg (active) erythromycin/kg body weight per day for 28 days (Table II-1). Unmedicated diets were fed to the control fish. A growth model based on the delta-I method of Butterbaugh and Willoughby (1967) was used to calculate the daily ration. Feeding schedules were adjusted according to actual growth and feed conversion data collected during bi-monthly samplings. A dry commercial salmon diet (Chinook AB salmon diet, Moore-Clark Canada, Inc., Vancouver, BC) was used throughout the study.

Commercially available non-sterile erythromycin phosphate (Abbot Laboratories, North Chicago, IL; 74.5% anhydrous potency) was used as the test drug. Medicated feeds were prepared by dissolving the antibiotics in 100% ethanol then spraying the mixture (25 ml/kg feed) onto the diet. The feed was mixed thoroughly and the ethanol allowed to evaporate at room temperature. Medicated diets were prepared weekly and stored at room temperature ($10 - 12^\circ\text{C}$) until used.

Mature fish were spawned during September and October 2001. Mature females were anesthetized (tricaine methanesulfonate, 50 mg/l), sacrificed by cervical dislocation, and the eggs removed through an incision extending from the vent to the pectoral fins along the ventral midline. Ovarian fluid was drained from the eggs before they were placed into individual, labeled plastic bags and stored on ice for up to 4 h before fertilization. Milt, collected by stripping from anesthetized fish, was stored on ice in separate oxygen-filled Whirl-pak® bags for up to 1 h before use.

Females were paired with males from the same pre-smolt treatment group (ES-1, ES-2, ES-3, AS-1, AS-2, or AS-3) so that the main and interactive effects of pre- and post-smolt treatment regimens could be evaluated. The fertilized eggs from each female were incubated in separate Heath-techna® downflow incubator trays supplied with pathogen-free well water (10°C , 12 l/min). At the eyed stage of development the eggs were shocked, counted and the percentage of viable (live) embryos determined. Unfed fry were weighed and counted at swim-up and samples collected for erythromycin analysis.

Collection and storage of tissues

Representative samples of gill, head and trunk kidney, liver, spleen, heart, pyloric stomach, pyloric caeca, upper and lower intestine, ovary, and brain were collected from all spawned fish and several immature fish from each treatment. The tissues were fixed in a freshly prepared solution of Davidson's fixative (30 parts 95% methanol/20 parts 40% formalin (paracide F)/10 parts glycerine/10 parts glacial acetic acid/30 parts distilled water). After 24 – 36 h, they were transferred to a solution of 70% (v/v) ethanol in distilled water and stored under refrigeration (5°C) until the tissues were trimmed and further processed. Additional samples of head and trunk kidney collected for erythromycin analysis were placed into individual Whirl-pak® bags and stored frozen (–20°C).

Histology

Prior to sectioning, the fixed tissues were routinely dehydrated and processed for embedment using a Shandon Hypercenter XP automated tissue processor, followed by embedment in Polyfin (Triangle Biomedical Sciences, Durham, N.C.). Tissues were sectioned at a thickness of 4 – 5 µm using a Reichert-Jung Biocut 2030 microtome. Sections were stained with hematoxylin and eosin-phloxine (Luna, 1968), and examined under a Nikon Optiphot light microscope. Approximately five fish per replicate tank (two replicate tanks per treatment) for a total of approximately ten fish per treatment from the most extreme treatment groups and control fish were evaluated for histopathology. Actual numbers of fish examined for histopathology in each treatment group were as follows: ES-3 x E-2 = 11; AS-3 x E-4 = 9; ES-2 x E-4 = 8; and ES-3 x E4 = 4; and controls = 14. All sections were examined by a single examiner with many years of experience in fish histopathology in a “blind” system in which the examiner was not aware of the treatment the fish had been subjected to until all specimens had been examined.

Antibiotic assays for erythromycin

The concentration of erythromycin in fish tissue was determined microbiologically using the bacterium *Micrococcus luteus*, which is sensitive to erythromycin concentrations as low as 0.0025 µg/10 µl of sample (Evelyn et al. 1986a). The method was modified from the agar diffusion method (Difco 1968; pp. 13-15) described by Evelyn and coworkers (1986a, 1986b). The plate base layer (45 ml of Difco antibiotic medium 11 (Difco Laboratories, Detroit, MI)) was poured into 150 x 15 mm sterile Petri dishes and over-laid with 13.5 ml Difco antibiotic medium 11 inoculated with 0.2 ml of a *Micrococcus luteus* culture (1.0 OD₅₄₀). Individual whole fish were placed in separate Whirl-pak® bags, weighed, then an (equivalent) volume of phosphate buffer solution (pH 7.2) was added to dilute samples. The samples were then homogenized with a rubber roller. Onto each inoculated plate, five sterile 7 mm paper disks (Becton Dickinson Microbiology System, Cockeysville, MD) were evenly placed around the plate, and one disk was placed in the center. Aliquots (10 µl) of the diluted homogenates were pipetted onto the disks. An aliquot of erythromycin the reference standard (2.0 µg/ml) was spotted on the center disk of each plate. Plates were incubated at 30°C for 24 h after which the diameter of the zones of inhibition were measured to the nearest 0.5 mm. The

antibiotic concentration in each test disk was calculated from a standard curve (inhibition zone diameter vs. \log_{10} antibiotic concentration in $\mu\text{g/ml}$) and corrected for dilution to determine equivalent antibiotic concentrations in the tissue ($\mu\text{g/g}$ wet weight).

Statistical methods

Statistical analysis of the experimental results was performed using JMP® statistical software (SAS Institute Inc., Cary, NC). The effect of post-smolt treatment frequency (percent) on survival was evaluated by single factor analysis of variance. The main and interactive effects of pre- and post-smolt antibiotic treatment regimens on fish weight at spawning, fecundity, egg weight, percentage of eggs surviving to the eyed stage, and average fry weight were evaluated by two factor analysis of variance. Fisher's PLSD was used for means separation when significant differences were detected ($p \leq 0.05$). Differences in prevalence of ovarian atresia among controls and the various treatment groups were tested by the Fisher's Exact Test, with critical level of significance set at $p \leq 0.05$. Differences in ordinal ratings of ovarian atresia severity among controls and treatment groups were tested by the nonparametric Kruskal-Wallis test, with critical level of significance set at $p \leq 0.05$.

Results and Discussion

Growth and survival

During Year 1, fish fed diets medicated with erythromycin exhibited adequate appetite during all feeding periods and growth was nearly identical among all treatment groups (Table II-2). Survival was uniformly high (>99%) and none of the signs of acute toxicity previously reported (hyperirritability, reddening of the skin) were observed. In August 2000, a severe acute mortality episode occurred in one replicate tank of E-4 fish immediately following a routine sampling during which all fish were individually weighed and measured. Sampling procedures were immediately modified to reduce handling stress (increased oxygenation, reduced time under anesthesia). These modifications were effective and only a few additional mortalities occurred in the remaining tanks of fish during the August 2000 sampling.

During Year 2, however, a clear trend of decreased growth and survival was observed in the erythromycin-treated groups (Table II-2). At the end of the experiment, the average weight of fish in the E-2 and E-4 groups was reduced by 9% and 18%, respectively, compared with the treatment controls. Nearly 92% of the control and 89% of the E-2 fish survived until the end of the experiment, compared with 69% those treated with erythromycin four times per year (E-4). Almost all mortality was directly associated with sampling-induced stress. Crowding elicited an extreme fright response characterized by rapid and erratic swimming. Moribund fish were observed within minutes of handling, and mortality continued for 12 – 24 h. No external signs of erythromycin toxicity were observed.

Reproductive effects

Oral administration of either azithromycin or erythromycin to fall chinook salmon one to three times during the first-feeding-to-smolt stage of development (ES and AS treatments in Study I) had no significant effect on weight of maturing adults, number of eggs produced, or gamete viability (Table II-3). In contrast, female weight, and both total and relative fecundity were significantly reduced by post-smolt feeding of erythromycin four, but not two, times per year.

Survival to the eyed egg stage, although substantially reduced by the E-4 treatment, was not significantly different from either the control or E-2 treatment groups. No interactive effects of pre-smolt and post-smolt treatment regimens on reproductive performance were found.

Histopathology

Lesions consistent with toxicity were not observed in the excretory or hematopoietic elements of the trunk kidney of fish from any of the erythromycin or azithromycin treatments or untreated controls; however, incidental findings of minor cases of nephrocalcinosis were observed in fish from the following treatments at the respective prevalences: untreated controls (2/14, 14.3%), and ES-3 x E-2 (2/11, 18.2%). No significant lesions were detected in liver, exocrine pancreas, gill, pyloric caeca, pyloric stomach, upper and lower intestine, heart, head kidney, or spleen. Sections of the brain in individual fish included portions of the medulla oblongata, cerebellum, saccus vasculosus, mesencephalon, optic tectum and optic nerves, olfactory lobe, and pituitary gland. No significant lesions were detected in any of the brain section components. Sections of the ovarian skein from females that had been manually spawned just prior to sacrifice and tissue collection were examined for presence and severity of ovarian atresia. Severity was ranked utilizing an ordinal scale ranging from 0 (absent) to 7 (severe). Atretic oocytes were typically yolked oocytes containing remnants of a thick, surrounding chorion. The respective prevalences and mean severities (\pm SD) of ovarian atresia in the controls and various treatment groups were as follows: untreated controls (7/14, 50%; 1.3 ± 1.5), AS-3 x E-4 (9/9, 100%; 3.3 ± 1.1), ES-3 x E-2 (11/11, 100%; 3.2 ± 1.5), ES-3 x E-4 (4/4, 100%; 2.7 ± 1.5), and ES-2 x E-4 (7/7, 100%; 3.1 ± 1.3). Prevalences of ovarian atresia were significantly higher than controls in the following treatment groups: AS-3 x E-4 ($p = 0.014$); ES-3 x E-2 ($p = 0.007$), and ES-2 x E-4 ($p = 0.029$). Although all females in the ES-3 x E-4 treatment group displayed ovarian atresia, the small sample size in this group ($n = 4$) precluded the determination of any significant differences from the control group prevalence. The severity of ovarian atresia was significantly higher in females from each of all four extreme treatment groups tested, as compared to controls by the Kruskal-Wallis test ($p < 0.01$). However, this test was unable to distinguish any significant differences in ovarian atresia among the four extreme treatment groups.

Antibiotic residues in treated adults and their progeny

At spawning, which took place 78 – 103 days after the final treatment was completed, erythromycin residues were detected in 17.1% and 57.0% of the female E-2 and E-4 fish

(Table II-4). The concentration of erythromycin in the kidneys of E-4 treated females ranged from 0.37 – 13.7 µg/g, compared with 0.56 – 4.08 µg/g in E-2 treated fish. There was no carry-over from any of the pre-smolt treatments to the adult fish.

In some cases, erythromycin was transferred to the progeny of mature adults through their eggs. Erythromycin was detected in the unfed fry of 34.7% (n = 17) of the mature E-4 females (n = 49) with detectable kidney levels of the antibiotic at spawning. Erythromycin concentrations ranged from 0.88 – 13.37 µg/g (5.34 ± 0.97 µg/g, mean ± SEM) in the kidney of mature females and 0.31 – 5.30 µg/g (1.45 ± 0.33 µg/g, mean ± SEM) in pooled samples of whole fry. Antibiotic residues were not detected in the progeny of any E-2 female.

Studies with trout and salmon have shown these species to be sensitive to the taste of erythromycin, and the effect increases with dietary concentration. Prescribed feeding rates decrease as fish grow, so the relative concentration of erythromycin must be increased proportionally to deliver the recommended daily therapeutic dosage. Chinook salmon smolts offered diets containing 6,700 mg/kg erythromycin thiocyanate consumed fewer pellets than those offered an unmedicated control feed (Schreck and Moffitt 1987). Furthermore, the number of fish that consumed more than one medicated pellet at each feeding declined as the antibiotic concentration increased to 10,000 and 12,000 mg/kg. In practical feeding trials, large (0.9 kg) rainbow trout (*Oncorhynchus mykiss*) initially offered a diet containing 8,300 mg/kg erythromycin at a rate of 1.2% of body weight per day, consistently rejected one-half of the daily ration (Warren 1963). Consequently, higher dietary antibiotic concentrations (ca. 16,700 mg/kg) were required to compensate for the lower ration level. In the present study, however, dietary erythromycin phosphate concentrations reached 15,321 mg/kg before reduced growth was observed.

In Year 2 of the study, dietary erythromycin concentrations were three to four times higher than the lowest level tested by Schreck and Moffitt (1987), and nearly 160% higher than that reported by Warren (1963). Although a trend toward reduced growth was observed in both treatments, significant differences occurred only in those treated four times per year, suggesting frequency of exposure may have also affected feed consumption. A similar effect was reported by Schreck and Moffitt (1987). In addition to affecting growth adversely, reduced consumption of medicated feeds may limit the therapeutic value of an orally-administered antibiotic. The depressed appetite of large fish approaching maturity, coupled with the effects of chronic BKD infections, may further compromise the effectiveness of treatment with erythromycin.

Symptoms of erythromycin toxicity observed in this study, including irritability and nervousness, a convulsive response to handling accompanied by exhaustion, morbidity, and mortality were similar to those previously reported in rainbow trout (Piper 1961, Warren 1963) and juvenile chinook salmon (Moffitt 1998). Erythromycin (100 – 500 mg/kg) was acutely toxic to rainbow trout within the first 3 – 6 days of treatment, but toxic effects were observed with chinook salmon only after prolonged oral therapy. In the present study, toxic effects were apparent only after four 28-day treatments with erythromycin (100 mg/kg) had been administered within a 12 month period. Fish treated

at longer intervals (6 months) were not affected, indicating orally-administered erythromycin has a relatively high toxicity threshold to chinook salmon, provided sufficient recovery time is given between treatments.

Histological examination revealed no deleterious changes in either the excretory or hematopoietic portions of the kidney, in contrast with the results of previous studies with rainbow trout chinook salmon. Both Piper (1961) and Wood (1963) reported hemoglobin deposition in the hematopoietic regions of the kidney of rainbow trout. Sulfa drug and antibiotic therapy have been frequently associated with pathological changes of this type (Wood and Yasutake 1955). Hemorrhagic ascites, yellow-green livers, and kidney damage that included tissue damage, necrosis and vacuolization of the tubules have been observed in maturing chinook salmon injected with erythromycin (Moffitt and Kiryu 2001). Hicks and Geraci (1984) reported necrotic damage to the proximal renal tubules of juvenile rainbow trout following oral administration of erythromycin (110 mg/kg). Cell damage was reversible 14 days after feeding ceased. Although adult fish in the present study were sampled 78 – 103 days post-treatment, recovery would not be expected because maturing fish are catabolizing tissues. Thus, substantial renal degeneration seems unlikely to have occurred in these fish.

Histological evaluation of ovarian tissues obtained from spawned females revealed significantly higher prevalence and severity of ovarian atresia in fish treated with erythromycin compared with the untreated controls. A corresponding trend toward reduced fecundity with increased treatment frequency, statistically significant only for the E-4 females, was also observed. Taken together, these data suggest that reductions in reproductive output in chinook salmon are linked with exposure to erythromycin, although the mechanism is unknown.

Female chinook salmon exposed to erythromycin four times annually had detectable erythromycin residues in their kidneys at spawning more than three times as frequently and at higher levels than females exposed only two times. The ability of orally-administered erythromycin to accumulate in fish tissues during treatment and to persist for prolonged periods after the end of treatment likely contributed to the observed differences. Moffitt and Schreck (1988) reported relatively high erythromycin concentrations in kidney of juvenile chinook salmon 19 days after 21 days of feeding at 50 mg/kg or 100 mg/kg (1.29 $\mu\text{g/g}$ and 2.16 $\mu\text{g/g}$, respectively). In the present study, the mean erythromycin concentrations in the kidney of E-2 and E-4 females were 0.89 and 2.63 $\mu\text{g/g}$, respectively, more than two months post-treatment, providing additional evidence that clearance follows a two compartment pharmacokinetic model, characterized by rapid early removal followed by a protracted, concentration-dependent terminal phase.

The transfer of orally-administered erythromycin from treated adults to their progeny has not been previously reported in the scientific literature. However, when erythromycin was injected into female chinook salmon at a total dose of 40 mg/kg, erythromycin residues were detected in kidneys (0.99 – 2.20 $\mu\text{g/g}$) and in egg vitellin (1.7 – 2.2 $\mu\text{g/ml}$) at spawning 28 days post-treatment (Hauknes and Moffitt 2002). Prespawning adult

salmon deposit injected erythromycin in their eggs (Evelyn et al. 1986b) where it may persist for up to 60 days before spawning. Bullock and Leek (1986) found detectable residues in 87% of the progeny of injected females 70 days post-spawning. In the present study, residues were detected in none of the progeny of the E-2 and 34.7% of the E-4 treated females that had erythromycin in the kidney at spawning. Variations in the initial concentration of erythromycin in the eggs, similar to those observed in kidney tissues, may partially explain these findings.

Reductions in fecundity, relative fecundity, and the percentage of eggs surviving to the eyed stage that occurred in chinook salmon given therapeutic dosages of erythromycin in their diets have important biological implications for broodstock programs in which endangered species are reared. Eyed egg production was reduced 8.4% by feeding erythromycin twice annually and 37% when two additional annual treatments were administered. It has become a routine practice to periodically administer erythromycin to chinook salmon broodstock at all phases of development to prevent mortality in fish presumptively infected with *R. salmoninarum*. In addition to reduced growth and survival, decreased gamete production and viability resulting from frequent exposure to erythromycin may substantially impact the effectiveness of rearing programs for ESA listed stocks. The results of this study underscore the need for developing and implementing strategies for preventing mortality due to bacterial kidney disease that minimize erythromycin usage.

Table II-1. Treatment schedule, fish sizes, and dietary concentrations of and erythromycin. Dietary antibiotic concentrations were adjusted based on actual feeding rates and feed conversion ratios to provide daily dosages of 100 mg/kg erythromycin.

Treatment period (days post-ponding)	Treatments	Average weight (g)	Dietary concentration (mg base/kg)
September 1999	E-4	59 – 86	5,986
December 1999	E-2, E-4	158 – 210	10,079
March 2000	E-4	265 – 338	12,066
June 2000	E-2, E-4	371 – 459	15,321
September 2000	E-4	508 – 586	19,900
December 2000	E-2, E-4	701 – 799	21,487
March 2001	E-4	988 – 1,112	23,813
June 2001	E-2, E-4	1,409 – 1,566	26,422

Table II-2. Effects of orally-administered erythromycin on average weight and survival (\pm SE) of fall chinook salmon. Duplicate groups of fish were treated either twice (E-2) or four (E-4) times per year for two years. Control groups did not receive antibiotic treatments at any time. Within-year means sharing a common superscript are not significantly different ($p < 0.05$, Fisher's PLSD).

Treatment	Year 1			<i>p</i>	Year 2			<i>p</i>
	Control	E-2	E-4		Control	E-2	E-4	
Average weight (g)	535.6 (1.1)	514.7 (14.8)	528.9 (28.5)	0.74	1,733.5 (52.8) ^a	1,583.3 (57.9) ^a	1,430.6 (22.0) ^b	0.045
Survival (%)	99.2 (0.4)	99.6 (0.4)	99.6 (0)	0.65	91.9 (2.3) ^a	88.6 (4.0) ^a	69.4 (3.6) ^b	0.032

Table II-3. Effects of orally-administered macrolide antibiotics on weight at spawning and reproductive performance of fall chinook salmon. Pre-smolt treatments were either azithromycin or erythromycin administered once, twice or three times beginning at ponding in January and ending at smoltification in May. Post-smolt treatments were erythromycin administered either two (E-2) or four (E-4) times per year for two years. Control groups did not receive antibiotic treatments at any time. Means (\pm SE) sharing a common superscript are not significantly different ($p < 0.05$, Fisher's PLSD).

	Post-smolt treatment			<i>p</i>		
	Control	E-2	E-4	Pre-smolt	Post-smolt	Interaction
Male weight	1,904 (64)	1,694 (60)	1,677 (122)	0.36	0.06	0.26
Female weight (g)	2,156 (58) ^a	2,086 (78) ^a	1,888 (46) ^b	0.79	0.03	0.61
Fecundity (eggs/female)	2,287 (77) ^a	2,094 (83) ^a	1,587 (67) ^b	0.96	<0.0001	0.66
Relative Fecundity (eggs/kg body weight)	1,073 (18) ^a	1,018 (16) ^a	853 (36) ^b	0.93	<0.0001	0.68
Egg weight (g)	0.170 (0.003)	0.174 (0.003)	0.182 (0.003)	0.64	0.08	0.54
Survival to eye (%)	79.3 (1.7)	79.3 (2.5)	71.8 (3.8)	0.62	0.15	0.77
Fry weight (g)	0.240 (0.003)	0.249 (0.005)	0.259 (0.006)	0.91	0.08	0.09

Table II-4. Antibiotic residues in kidney tissue from mature female fall chinook salmon treated either two (E-2) or four (E-4) times per year for two years. The final treatment period ended approximately two months prior to sampling.

Treatment	Number of fish tested	Detection rate (%)	[Erythromycin] ($\mu\text{g/g} \pm \text{SE}$)
E-2	140	17.1	0.89 (0.18)
E-4	86	57.0	2.63 (0.45)

Data Management Activities

Data are collected by NOAA and PSMFC researchers onto preformatted data sheets or directly into personal computers. Data are entered and summarized on personal computers operated by researchers using programs such as Excel, Filemaker Pro, GraphPad Prism, and JMP statistical software (for complete list of methods see the Materials and Methods section of each experimental section). All data are checked for quality and accuracy before and after analysis. Analytical processes are described in the text of the annual report under Materials and Methods. Data analyses are reported in the Results.

Summary and Conclusions

Fall chinook salmon obtained as unfed fry in January 1999 were reared in pathogen-free well water (10°C) until they matured in September 2001. The accumulation and clearance of orally-administered azithromycin and erythromycin in juvenile fish was evaluated through the smolt stage, and the effects of long-term administration of erythromycin on their reproductive performance were studied. In the fry-to-smolt phase (see Study I), duplicate groups of fall chinook salmon (initial weight 0.42 g) were pair-fed diets medicated with azithromycin (target dosage, 30 mg/kg for 14 days) or erythromycin (target dosage, 100 mg/kg for 28 days) either one (AS-1, ES-1), two (AS-2, ES-2), or three times (AS-3, ES-3) beginning two weeks after first-feeding (February 1999) and ending at smoltification (June 1999). Unmedicated diets were fed between treatments, and to the experimental control (E-0). Growth, antibiotic accumulation and depletion, and toxic effects were evaluated.

Fish from each primary treatment were PIT-tagged and redistributed so secondary treatments (no additional treatment (E-0), or erythromycin (target dosage, 100 mg/kg for 28 days) fed either two (E-2) or four (E-4) times per year for two years) could be applied

in the smolt-to-adult phase of the trial (Study II). Treatment effects on growth and survival, adult size, fecundity, and gamete quality were measured.

In summary, medicated diets were well accepted by juvenile fall chinook salmon, and no palatability problems were observed. Growth of treated and control fish was similar except during the final treatment period when reduced growth was observed in the treated groups.

Erythromycin was poorly absorbed by first-feeding fry, but increased with fish size. Whole body concentrations averaged less than 0.2 µg/g in first-feeding fry and about 10 µg/g at smoltification. Erythromycin was cleared from the tissues within three weeks post-treatment. No signs of toxicity were observed.

Azithromycin was concentrated in the tissues to a greater extent than was erythromycin during all feeding periods, at all treatment frequencies. Whole body concentrations averaged 18.98 µg/g in first-feeding fry and nearly 50 µg/g at smoltification. Azithromycin was detected in the tissues of chinook salmon smolts 74 days after feeding ended. No signs of toxicity were observed.

During the smolt-to-adult phase of the study, E-4 fish were highly sensitive to handling, exhibiting nervousness and elevated mortality. Overall survival was 89 – 92% in the E-0 and E-2 groups, compared with 69% in the E-4 treatment. Growth was depressed in both treated groups. At the end of the trial, control fish averaged 1,733 g, compared with 1,583 g and 1,431 g in the E-2 and E-4 groups, respectively.

Eyed egg production was reduced 8.4% by feeding erythromycin twice annually and 37% when two additional annual treatments were administered.

Erythromycin residues were detected in 57% of E-4 female spawners, compared with 17% of those in the E-2 group.

Carryover from some E-4 (but not E-2) females to the fry was also observed. Kidney tissue concentrations averaged 5.34 µg/g in spawned females, and 1.45 µg/g in their progeny.

The effects of erythromycin on reduced growth and survival, reductions in fecundity and relative fecundity, and the percentage of eggs surviving to the eyed stage that occurred in chinook salmon given therapeutic dosages of erythromycin in their diets have important biological implications for broodstock programs in which endangered species are reared. The results underscore the need for developing and implementing strategies for preventing mortality due to bacterial kidney disease that minimize erythromycin usage.

References

- Bandín, I., Y. Santos, A.E. Toranzo, and J.L. Barja. 1991. MICs and MBCs of chemotherapeutic agents against *Renibacterium salmoninarum*. *Antimicrob. Agents Chemother.* 35(5):1011-1013.
- Bullock, G.L., and S.L. Leek. 1986. Use of erythromycin in reducing vertical transmission of bacterial kidney disease. *Vet. Hum. Toxicol.* 26(1):18-20.
- Butterbaugh, G.L., and H. Willoughby. 1967. A feeding guide for brook, brown, and rainbow trout. *Prog. Fish-Cult.* 29(4):210-215.
- Evelyn, T.P.P., J.E. Ketcheson, and L. Prosperi-Porta. 1986a. Experimental intra-ovum infection of salmonid eggs with *Renibacterium salmoninarum* and vertical transmission of the pathogen with such eggs despite their treatment with erythromycin. *Dis. Aquat. Org.* 1:197-202.
- Evelyn, T.P.P., J.E. Ketcheson, and L. Prosperi-Porta. 1986b. Use of erythromycin as a means of preventing vertical transmission of *Renibacterium salmoninarum*. *Dis. Aquat. Org.* 2:7-11.
- Flagg, T.A., W.C. McAuley, D.A. Frost, M.R. Wastel, W.T. Fairgrieve, and C.V.W. Mahnken. 2000. National Marine Fisheries Service, Redfish Lake Sockeye Salmon Captive Broodstock Rearing and Research, 1995-2000, Annual Report 2000, Report to Bonneville Power Administration, Contract 1992BP41841, Project No. 1992294000, 71 electronic pages (BPA Report DOE/BP-41841-4).
- Fryer, J.L. 1987. Department of Microbiology, Epidemiology and Control of Infectious Diseases of Salmonids in the Columbia River Basin, Annual report 1987 to Bonneville Power Administration, Portland, OR, Contract 83-AI-11987, Project 38-312, 130 electronic pages (BPA Report DOE/BP-11987-3).
- Groman, D.B. 1983. Studies examining the identification, epizootiology, and control of *Renibacterium salmoninarum* infections in chinook salmon (*Oncorhynchus tshawytscha*). Doctoral dissertation. University of Idaho, Moscow, Idaho.
- Gutenberger, S.K., J.R. Duimstra, J.S. Rohovec, and J.L. Fryer. 1997. Intracellular survival of *renibacterium salmoninarum* in trout mononuclear phagocytes. *Dis. Aquat. Org.* 28:93-106.
- Hauknes, A.H., and C.M. Moffitt. 2002. Hatchery evaluation of erythromycin phosphate injections in prespawning spring chinook salmon. *N. Am. J. Aquacult.* 64:167-174.
- Hicks, B.D., and J.R. Geraci. 1984. A histological assessment of damage in rainbow trout, *Salmo gairdneri* Richardson, fed rations containing erythromycin. *J. Fish Dis.* 7:454-465.
- Luna, L.B. 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology, 3rd edition. McGraw-Hill, New York, New York. 258 pp.
- Moffitt, C.M. 1998. Field trials of investigational new animal drugs. *Vet. Hum. Toxicol.* 40(2):48-51.
- Moffitt, C.M., and Y. Kiryu. 2001. Acute and chronic toxicity following parenteral application of erythromycin to maturing spring chinook salmon held at two water temperatures. *J. Aquat. Anim. Health* 13:8-19.

- Moffitt, C.M., and J.A. Schreck. 1988. Accumulation of orally-administered erythromycin thiocyanate in the tissues of chinook salmon. *Trans. Am. Fish. Soc.* 117:394-400.
- Nagahama, Y. 1983. The functional morphology of teleost gonads. *In* W.S. Hoar, D.J. Randall, and E.M. Donaldson (editors). *Fish Physiology*, Vol. 9A, pp. 223-275. Academic Press, New York.
- Peters, D.H., H.A. Freidel, and D. McTavish. 1992. Azithromycin. A review of its antimicrobial activity, pharmacokinetic properties, and clinical efficacy. *Drugs* 44:750-799.
- Piper, R.G. 1961. Toxic effects of erythromycin thiocyanate on rainbow trout. *Prog. Fish-Cult.* 23:134-137.
- Schiewe, M. H., T.A. Flagg, and B.A. Berejikian. 1997. The use of captive broodstocks for gene conservation of salmon in the western United States. *Bull. Natl. Res. Inst. Aquacult., Suppl.* 3: 29-34.
- Schreck, J.A., and C.M. Moffitt. 1987. Palatability of feed containing different concentrations of erythromycin thiocyanate to chinook salmon. *Prog. Fish-Cult.* 49(4):241-247.
- Sneiszko, S.F., and E.M. Wood. 1955. The effect of some sulfonamides on the growth of brook trout, brown trout, and rainbow trout. *Trans. Am. Fish. Soc.* 84:86-92.
- Warren, J.E. 1963. Toxicity tests of erythromycin thiocyanate in rainbow trout. *Prog. Fish-Cult.* 25:88-92.
- Wolf, K., and C.E. Dunbar. 1959. Tests of 34 therapeutic agents for control of bacterial kidney disease in trout. *Trans. Am. Fish. Soc.* 88:117-124.
- Wood, J.E., and W.T. Yasutake. 1955. Sulfonamide toxicity in brook trout. *Trans. Am. Fish. Soc.* 84:155-161.