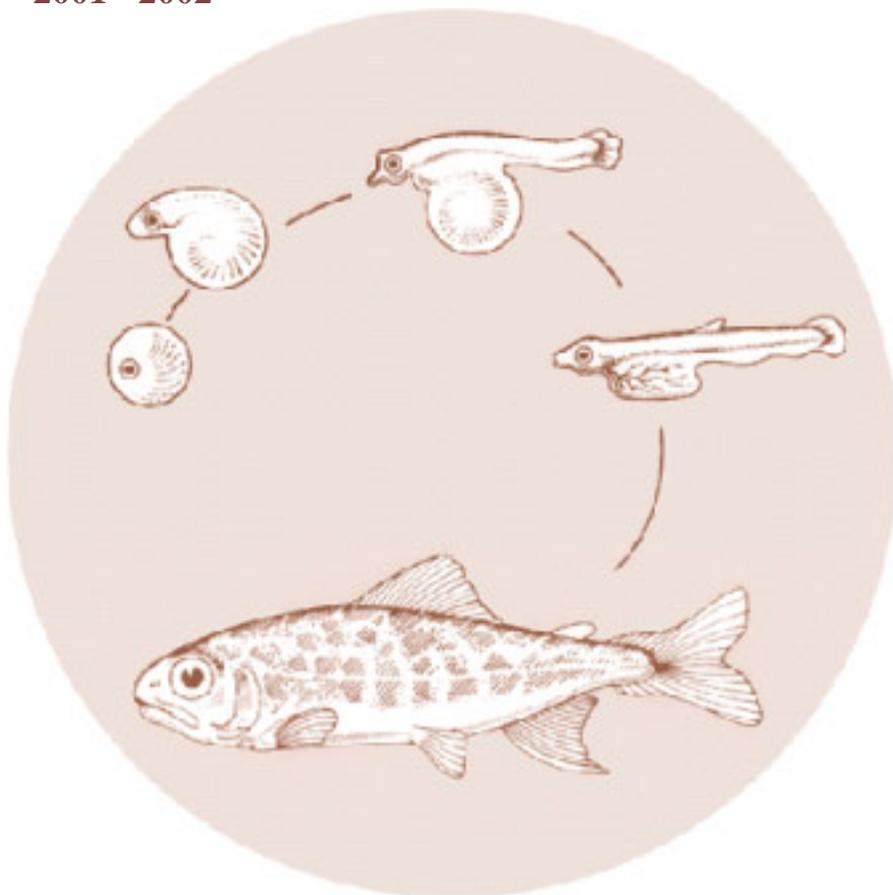


Research on Captive Broodstock Programs for Pacific Salmon

Annual Report
2001 - 2002



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RESEARCH ON CAPTIVE BROODSTOCK PROGRAMS FOR PACIFIC SALMON

**ANNUAL REPORT
(PERFORMANCE PERIOD: 1 JUNE, 2001 THROUGH 31 MAY, 2002)**

Prepared by

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EXECUTIVE SUMMARY

The efficacy of captive broodstock programs depends on high in-culture survival and the fitness 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. The following summarizes some of the work performed and results from the FY 2001 performance period:

- The incidence of male maturation of age-1 chinook salmon was significantly reduced by reducing growth in the first year of rearing.
- Experimentally manipulated growth rates of captively-reared coho salmon had significant effects on female maturation rate, egg size, and fecundity, and the effects were stage-specific (i.e., pre-smolt vs. post-smolt).
- A combination of Renogen and MT239 vaccination of yearling chinook salmon given an acute *R. salmoninarum* challenge had a significantly longer survival time than the mock-vaccinated group. The survival time was marginally higher than was seen in acutely challenged fish vaccinated with either Renogen or MT239 alone and suggests that a combination vaccine of Renogen and MT239 may be useful as both a prophylactic and therapeutic agent against BKD.
- Full-sib (inbred) groups of chinook salmon have thus far exhibited lower ocean survival than half-sib and non-related groups. Effects of inbreeding on fluctuating asymmetry did not follow expected patterns.
- Sockeye salmon were exposed to specific odorants at either the alevin/emergent fry stage or the smolt stage to determine the relative importance of odorant exposure during key developmental periods and the importance of exposure duration,
- Experimental studies to determine the effects of exercise conditioning on steelhead reproductive behavior and the effects of male body size on chinook salmon fertilization success during natural spawning were completed.

REPORT STRUCTURE

This report is organized by Task number as outlined in the FY 2001 Statement of Work. For each Task there is included either a Final Report or a Progress Report.

Final Reports include data presentation, statistical analysis, and an interpretation (i.e., discussion) of the results for the performance period indicated. Final Reports are not meant to imply that the research has been completed, only that the reporting for the noted performance period is complete. In many cases, further research on the same or similar topics may be necessary and continued in future years under this project.

Progress Reports¹ have been included for Tasks that extend beyond the performance period, or for experiments in which data analysis has not been completed. In most Progress Reports, preliminary data have been excluded purposely to eliminate potential problems with misinterpretation of incomplete data sets and analyses. Final Reports for these Tasks will be completed according to the schedules described in the Statement of Work.

¹ Final reports for Tasks 7 and 8 (principal investigator: Ron Hardy, University of Idaho) will be completed by June 2003 and will be included in the FY02 Annual report. They are not included in the FY01 Annual Report.

PEER-REVIEWED PUBLICATIONS OF THE PROJECT

Published Papers

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

(PROGRESS REPORT: 1 JUNE, 2001 THROUGH 31 MAY, 2002)

by

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Introduction

In the 2000 Federal Columbia River Power System (FCRPS) Biological Opinion, NMFS identified six populations of steelhead and several salmon populations that had dropped to critically low levels and continue to decline. Following thorough risk-benefit analyses, captive propagation programs for some or all of the steelhead (*Oncorhynchus mykiss*) 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 the other objectives of BPA Project 1993-056-00 currently underway for chinook (*O. tshawytscha*) and sockeye salmon (*O. nerka*).

Pacific salmon reared to the adult stage in captivity exhibit poor reproductive performance when released to spawn naturally. Poor fin quality and swimming performance, incomplete development of secondary sex characteristics, changes in maturation timing, and other factors may contribute to reduced spawning 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.

Previous NMFS studies have evaluated the breeding behavior and reproductive success of captively-reared coho (*O. kisutch*) 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. *in press*). In chinook salmon, direct comparisons have not been made between captively-reared and wild adults; however, several anomalous behavior patterns have been observed. For example, females frequently abandoned their nests and, after several hours of continuous courtship and numerous male ejaculations, never returned to spawn. In the same study, sexually mature males often showed little or no courtship behavior when nearby nest-digging females were present (Berejikian et al. 2000). In addition, the eggs of adult captively-reared chinook salmon spawning in natural streams in Idaho suffered mortality much greater than observed in captively-reared fish spawned artificially (P. Kline, IDFG, pers. commun.). These studies indicate that captive rearing causes reproductive behavioral deficiencies in Pacific salmon.

To guide future decisions on captive broodstock reintroduction strategies and improve husbandry practices, it is critical to determine whether: 1) captively-reared steelhead reproductive performance is compromised to the extent reported for chinook salmon (Berejikian et al. 2000, 2001), and 2) reproductive performance can be improved by modifying rearing protocols. Task 1 evaluates the effect of current velocities in rearing tanks on reproductive behavior, and adult-to-parr reproductive success of steelhead.

A large body of published literature suggests that exercise positively influences numerous 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). In 2000 a study was conducted with captively-reared chinook salmon to determine the effects of exercise (current velocities increased to approximately 0.6 body length/s) on reproductive performance. Preliminary results suggest that exercised females spawned more nests and covered their nests with greater frequency than non-exercised females. Exercise had no effect, however, on onset of spawning or egg deposition, which are two primary factors limiting the reproductive success of captively-reared salmon. Thus, in chinook salmon exercise probably provided a marginal benefit during spawning. The chinook salmon were exercised for approximately 5 months during seawater rearing, then exercised and non-exercised fish were combined into low velocity freshwater vessels for approximately 2 months prior to final maturation. The duration and timing of exposure to a high velocity may have been inadequate to affect characteristics related to spawning. In continuation of Task 5 from the FY 2000 statement of work, steelhead that had been reared in replicate tanks were evaluated for the effects of 2.5 years of continuous exercise on reproductive behavior and success.

Work Completed

A subsample of the eyed embryos ($n = 304$) collected from the Hamma Hamma River in 1998 were allocated to a rearing and reintroduction strategy that included rearing the fish to sexual maturity prior to release for natural spawning in the Hamma Hamma River in 2002. In May 1999, 296 yearlings were divided equally into four circular rearing tanks (6 m in diameter) at the Lilliwaup Hatchery. The Lilliwaup Hatchery, operated by Long Live the Kings (a non-profit organization), is located near the confluence of Lilliwaup Creek and Hood Canal, approximately 15 km from the Hamma Hamma River. Pumps (2.0 HP) and variable speed controllers were emplaced in two tanks to re-circulate water and create current velocities up to 0.40 m/sec. Velocities in these two tanks were increased to approximately 1 body length/s (BLPS) for 12 hours each day between 22 October 1999 and 27 March 2000, after which velocities were increased to the same velocity for 23 hours/day. No adverse behavioral effects on the fish were observed as a result of the near-continuous exercise. The fish were measured every 3 months, and the pumps adjusted to maintain 1 BLPS for 23 hours/day. Velocities in the control tanks were maintained at approximately 0.25 BLPS. Fish in all tanks were fed similar rations. Each fish was injected with a PIT tag for individual identification during rearing and up to the point of external tagging.

Breeding behavior and success evaluations

A subsample of 64 adults (32 males and 32 females) was transported to the NMFS Manchester Research Station (MRS) on 23 February 2002, and the fish were acclimated in 5-m diameter circular tanks. Each fish was anesthetized, weighed, measured, and an individually numbered Petersen Disk Tag (2.5 cm) was inserted for individual identification. Mature fish were released into an experimental spawning channel at the MRS on 25 February 2002. The channel was divided into two replicate sections, each measuring 40 m long by 3 m wide, and each containing eight contiguous 5-m long by 3-m wide sections separated by 15-cm water fall. Each section received 6 males and 6

females from each of the four tanks (24 fish total). The stream channel had water depths (25 - 35 cm), velocities (up to 0.5 m/s), gravel size (1 - 10-cm diameter), and temperatures (approximately 6.0 to 10°C) 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 temperature between 6.0 and 9.5°C throughout spawning period.

Fish in the stream channel were observed from an observation blind each day between 0700 and 1900 h from early March through June 2002. All fish in each of the 16 sections were observed for two or three 10-min periods each day. Behaviors were recorded by scan (5-minute) sampling twice daily for each fish in the channel. Several behavioral characteristics important in determining breeding success were measured. Male breeding success is largely determined by their competitive ability, access to sexually active females, courtship behavior, and breeding longevity (Fleming and Gross 1994). In females, onset of spawning, competition for nest sites, egg deposition, and nest guarding ability are primary factors affecting breeding success (Fleming and Gross 1994). Behaviors recorded (Table 1) were defined by Tautz and Groot (1975) for rainbow trout.

Table 1. Behaviors recorded for female and male steelhead.

Females	Males
Nest digging frequency	Quiver frequency
Spawning frequency (number of nests)	Crossover frequency
Cover digging frequency	Spawning as dominant male
Onset of spawning	Spawning as secondary male
Spawning duration	Attacks
Attacks	Threat displays
Threat displays	

During each 10-min observation period, the reproductive status of each male was categorized as either 'courting,' 'satellite,' 'wandering,' or 'inactive.' Courting males attend females and exhibit courtship behaviors, including crossovers and quivers. Satellite males are closely associated with the courting pair, but positioned slightly downstream. Satellites have frequent aggressive interactions but are subordinate to the dominant (courting) male. Males actively swimming or holding position in fast currents, but not competing for access to or courting a sexually active female, were described as wandering. Males largely quiescent in the stream margins were described as inactive.

Work to be completed

Data analysis

Between-treatment comparisons will be made for each sex. For males, quiver, crossover, attack and threat display frequencies will be compared by ANCOVA with body size as the covariate. Contingency table analysis (Chi-square) will be used to determine whether males from the two treatments participated in spawning events as the

dominant and satellite male at unequal frequencies. The proportion of observations in which males were observed in each status will be calculated, arcsin-transformed, and compared by a two-sample t-test.

For females, nest digging, cover digging, attack and threat display frequencies, onset of spawning, and spawning duration will be compared by ANCOVA with body size as the covariate. The estimated number of eggs deposited by each female will be calculated by subtracting the number of eggs retained in the body cavity (determined by dissection) at death from their estimated fecundity. The fecundity of each female used in the spawning experiment will be estimated by entering their length into length-fecundity regression equations developed from artificially spawned females not used in the spawning study. Egg deposition will be compared between the treatments by ANCOVA (body size covariate).

The FY02 Statement of Work describes the continuation of this Task, which includes monitoring fry emergence, emigration, and growth. DNA pedigree analyses will be conducted to quantify adult-to-parr reproductive success, and determine maternal and paternal effects of parr movements, emigration, and growth. Work conducted during the FY01 and FY02 performance periods will be combined, and a manuscript prepared for publication by June 2003.

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**TASK 2. DETERMINE THE RELATIVE FERTILIZATION SUCCESS OF
CHINOOK SALMON FEMALES PAIRED WITH LARGE (AGE 4) VERSUS
SMALL (AGE 3) MALES.**

(PROGRESS REPORT: 1 JUNE, 2001 THROUGH 31 MAY, 2002)

by

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Introduction

Studies conducted in 1999 and 2000 by the IDFG found that a large proportion (>75%) of eggs from captively-reared chinook salmon females deposited in natural streams do not produce viable embryos. The poor viability may have been caused by: i) poor gamete quality, ii), inadequate incubation environment caused by poor nest construction or location, or iii) poor fertilization caused by asynchronous timing of gamete release. Fertilization success and embryo viability are generally greater than 70% in artificially-spawned captively-reared chinook salmon, reducing the likelihood that gamete quality is the primary reason for poor viability in naturally spawning fish. Therefore, the two most likely causes for poor egg viability in naturally spawning captively-reared chinook salmon are inadequate incubation environment caused by poor nest construction and poor fertilization success. The present Task was completed to test the possibility of poor fertilization success, primarily because as many as 12% of eggs sampled from individual nests of captively-reared females have been verified to be unfertilized, and a recent study suggests that courtship behavior between large (age-4) females and smaller (age-3) males (a common mating combination in captively reared populations) may be compromised (Berejikian et al 2000). Fertilization rates in naturally spawning wild populations have been consistently greater than 90% in the same Idaho streams (P. Kline, IDFG, unpublished data).

Fertilization success of mature male parr and jack males acting as secondary males, or 'sneaks,' is quite variable, ranging between 3-93% in sockeye salmon jacks (Foote et al. 1997) and 0-77% in Atlantic salmon mature male parr (Mjølnerod et al. 1998). However, neither these nor other studies have investigated the fertilization success of jack males acting as the lone dominant (courting) male. This situation is frequently encountered in captively-reared populations of chinook salmon, because of early (ages 2 and 3) male maturation resulting in few same-age males available for release with cohort females. Berejikian et al. (2000) found that chinook salmon females delayed spawning in the presence of smaller males, but eventually deposited nearly as many eggs when courted by small males as they did when courted by large males. However, the significant positive correlations between female nest construction behaviors and male courtship behaviors were only detected when females were paired with large males. Thus, the hypothesis is that synchronized gamete release, which requires reciprocal stimuli between the male and female (de Guademar and Beall 1999), may not be occurring in size-mismatched pairs, thereby reducing fertilization success.

The present Task was undertaken to determine the fertilization success of females paired with either large or small males. Fertilization rates of naturally spawning fish were determined by sampling eggs hydraulically at the eyed stage of development. The same fish used in the spawning experiment were also artificially spawned to separate behavioral effects from differences in gamete quality.

Work Completed

Maturing chinook salmon were obtained from Big Beef Creek, near Seabeck, WA. Sixteen maturing females were released into replicate 15-m² sections of the MRS stream channel described in Task 1. Males were introduced within 12 hours after a female had begun to dig a nest. Females were paired with either a large male (equal in

weight) or a small male (0.3 - 0.5 x female weight). The behavior of each pair was monitored 24 hours a day by infrared sensitive cameras positioned above the stream channel. Time-lapse recordings were made to ensure that all spawning events were documented. In addition, underwater cameras connected to VCR recorders recorded the behavior of each pair spawning during the day.

Approximately 150 eyed eggs were removed from each nest in which eggs were deposited by the following method. Within 1 minute after each day-time spawning had occurred a metal weight with an attached piece of nylon flag was deposited into the center of the nest pocket for later identification. Accumulated temperature units were monitored to determine when the eggs in each nest had reached eyed stage of development. Eyed eggs were removed hydraulically by pumping water into the nest pockets, which forced eggs into the water column and into a bag seine that the nest.

Each pair of chinook salmon were removed after having spawned twice. The pairs were immediately spawned artificially. The fertilized eggs were placed into replicate 15-cm diameter isolletes (small circular incubators), which were randomly placed in Heath tray incubators. At the eyed stage of development, artificially spawned eggs were 'shocked' to determine the number of viable and non-viable embryos. Non-viable embryos that were produced from the artificial and natural spawnings were 'cleared' with Stockard's solution and inspected to determine the presence or absence of cell division, which would indicate whether or not fertilization occurred.

Work to be completed

Day and night-time video tapes will be decoded and data analyzed. An observer will record from the video tapes the frequency of known courtship and nest construction activities by the male and female (such as crossovers, quivers, nudges, digs, and probes) and enter the information into a computer using the Observer program (Noldus V. 4.0). The following response variables will also be recorded at the precise time of spawning: i) relative timing of gamete release, ii) latency to male nest entry, iii) position of the male relative to the female, and iv) duration of gamete release for each sex.

The relationship between fertilization of eggs from artificial and natural spawning within a pair will be tested by linear regression analysis. Residuals of the natural-artificial spawning regression will be calculated. The residuals will be considered to represent variation in fertilization success not caused by variation in gamete quality. The residuals will therefore be used as the dependent variable in a repeated-measures ANOVA to compare the fertilization success of large males with small males (main effect), with nest number as the repeated measure. Relationships between fertilization success and behavioral measures before and during spawning will also be explored.

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**TASK 3. EVALUATE AND MONITOR THE BREEDING BEHAVIOR AND
SUCCESS OF ESA-LISTED CAPTIVELY-REARED CHINOOK SALMON IN
IDAHO STREAMS**

by

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Introduction

Release of captive-reared adult chinook salmon is the main reintroduction strategy for three populations of threatened chinook salmon in Idaho. Monitoring of adult releases in 1998 - 2000 has revealed captive-reared chinook undergo final maturation approximately 3 – 5 weeks later than wild fish. The success of this release strategy depends on natural maturation timing to allow for intermixing of captive-reared and wild chinook salmon, and natural emergence timing of the offspring. Monitoring of actual reintroductions of ESA-listed stocks is a critical component of captive broodstock programs. Studies on chinook salmon breeding behavior have been published by NMFS (e.g., Berejikian et al. 2000, Berejikian et al. 2001; see references in Task 1) as a result of this project. NMFS expertise in evaluating breeding behavior and success of spawning salmon was applied to improve the effectiveness of reintroduction monitoring efforts in Idaho streams, as carried out between 1999 and 2001.

IDFG implemented an acclimation program in an attempt to achieve natural maturation timing. Captively-reared chinook salmon from the West Fork Yankee Fork (WFYF) Salmon River were transferred from the MRS to the IDFG Eagle Hatchery in May 2002. Half of the maturing fish were held in a tank supplied with 14°C well water (ambient) and the other half were held in chilled 9°C well water until release into the WFYF River.

Methods

The main objective was to determine the onset of sexual activity (nest digging in females and courtship in males) of chinook salmon from the two acclimation temperature regimes (9°C and 14°C). The NMFS contributed approximately two weeks' assistance to IDFG efforts, one week for planning and one week of direct monitoring and information transfer.

All adult fish were measured, weighed, and identified by PIT tags as part of the ongoing IDFG pre-release sampling program. A colored, numbered anchor tag (Floy Tag Company) was added before fish were released into the stream(s) for natural spawning.

Each of six stream sections (~ 1.5 km each) in the WFYF River were scanned at least once per week. The location of each fish observed was recorded and GPS coordinates assigned. The reproductive status of males and females was recorded using the same protocols as described in Task 1. When a courting pair was observed the frequencies of aggressive and courtship behaviors were continuously recorded in 10-minute increments prior to, during, and after spawning.

Results

Data collected by NMFS were submitted to IDFG, which will summarize and analyze all the information. NMFS will contribute to data analysis and reports as requested by IDFG.

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**TASK 4. THE EFFECTS OF GROWTH RATE/SIZE ON THE INCIDENCE OF
EARLY SEXUAL MATURATION IN MALE SPRING CHINOOK SALMON
(*ONCORHYNCHUS TSHAWYTSCHA*)**

(FINAL REPORT: 1 JUNE 2000 THROUGH 31 MAY 2002)

by

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Introduction

Male chinook salmon (*Oncorhynchus tshawytscha*) have a plastic life history and can sexually mature between 0 to 7 years of age. Early sexual maturation (i.e., maturation before the youngest females) occurs in wild males but the incidence is far lower than observed in hatchery-reared fish (Foote et al. 1991, Mullan et al. 1992). Up to 96% of male spring chinook salmon have been reported to mature early when fish are captively-reared (Hard et al. 1985). There are currently eight stocks of spring chinook salmon that are listed as Threatened and are being reared in captivity in the Pacific Northwest. In addition, there are approximately 400 million chinook salmon produced annually by hatcheries for liberation into the North Pacific Ocean. For captively-reared stocks, females generally mature at 4 or 5 years of age, while males frequently mature at 1 or 2, resulting in a loss to the gene pool when inter-cohort crosses are desirable. These losses are variable but can exceed 15% of the one-year-old males and a further 30% at age 2 (C. McAuley, NMFS, pers. commun.). The extent of the losses at supplementation hatcheries from early male maturation is unknown, as these fish are generally released from the hatchery before the latter stages of sexual maturation.

Age of sexual maturation of male chinook salmon is influenced by genetic, biotic and abiotic factors (Power 1986). The level of energy stores has been shown to influence the incidence of sexual maturation in Atlantic (Rowe and Thorpe 1990b) and Pacific salmon (Silverstein et al. 1997, Shearer and Swanson 2000) but fish size and/or growth rate at specific times of the year appear to be the biotic factor exerting the most influence in chinook salmon (Hopkins and Unwin 1997, Silverstein et al. 1997, Shearer et al. 2000). Data so far suggest that maturation is initiated in the late fall or winter, approximately 10 months prior to spawning (Silverstein et al. 1997, Shearer and Swanson 2000, Shearer et al. 2000). Studies in Atlantic salmon suggest that spring is a permissive period when maturation will continue if growth and energy acquisition are adequate during the spring prior to spawning. Reducing feeding level or fasting during this spring period has been shown to reduce the incidence but not prevent early male maturation in Atlantic (Rowe and Thorpe 1990a, Herbinger and Friars 1992, Berglund 1995) and chinook salmon (Hopkins and Unwin 1997). The optimum strategy for preventing early maturation, therefore, appears to be to prevent initiation. The primary objective of the experiment was to determine if there is a threshold size for early male maturation. This was accomplished by raising fish on six different rations of a commercial feed. In addition, physiological samples were collected 10 months prior to spermiation to determine if maturation had commenced.

Materials and Methods

Eggs from the Willamette salmon hatchery were obtained in October 1999 and incubated at 5°C at the National Marine Fisheries Service facilities in Seattle. At first feeding in February 2000, the fish were placed in 1.3-m diameter tanks at either 5°C (treatments 1-5) or 8°C (treatment 6) (Figure 1). In April, when average fish weights were either 0.9 g (for those reared at 5°C) or 1.3 g (for those at 8°C), fish were transferred into experimental tanks. The fish in treatment 6 were reared at a higher

temperature than fish in the other treatments until August to accelerate their growth (Figure 1). At this time the fish in treatment 6 were moved to 2.6-m diameter tanks. Using the bioenergetics model of Cho (1992), a daily ration was calculated to produce fish of 10, 15, 20, 25, 50 and 110 g (treatments 1-6) on 1 December 2000. The model requires inputs for the following: rearing temperature, the thermal growth coefficient (TGC; 44, 65, 75, 84, 92, 126×10^{-5} for diets 1-6), 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 were fed the same commercial feed (BioOregon Grower, 22.1 KJ/g energy, 49% protein, 22% lipid). Initial tank stocking densities were 600 fish per tank and there were two replicate tanks per treatment. The experiment was terminated in July 2001, 550 days after first feeding, when maturation could be confirmed by the size of the testes.

Ten fish from each tank were randomly selected for weight, length, and body composition determination approximately monthly over the first year and approximately every six weeks thereafter. At the end of the experiment, the remaining fish were 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 subsample 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. Energy density of feeds was determined using an oxygen combustion bomb (model 1108, Parr Instrument Co., Moline, IL.).

On 18 December (day 352), 60 fish were sampled for the measurement of endocrine and cellular markers of maturation. Treatment groups 2, 4, and 6 were sampled (30 fish from each replicate tank). As these groups were mixed sex, 24 to 31 males per treatment were sampled. Fish were killed in a lethal dose of anesthetic, weighed and measured. Blood was collected via the caudal vein using heparinized syringes. Testes were fixed in Bouin's fixative for 24 hours prior to storage in 70% ethanol. Bodies were stored at -20°C for proximate analysis (see previously). Blood was centrifuged at 3000 x g for 3 minutes and plasma stored at -70°C.

Plasma 11-ketotestosterone (11-KT) was measured according to Schulz (1984), using a methylene chloride extraction method described in Planas and Swanson (1995). Plasma insulin-like growth factor (IGF-I) was measured using GroPep components as described by Shimizu et al. (1999). Fixed testes were dehydrated through ethanol and imbedded in paraplast, sectioned, and stained with hematoxylin and eosin. Stages of spermatogenesis were determined by light microscopy using Schulz (1984) as a guide. The presence of late type-B spermatogonia was used as a criterion to identify testis-initiating-maturation as this cell type appears just prior to initiation of meiosis.

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

The growth of the fish closely followed the weights predicted using our feeding protocol. The mean weights of the fish in treatments 1-6, based on the replicate tank means, were 10.9 ± 1.3 , 16.4 ± 0.4 , 19.3 ± 0.8 , 22.1 ± 1.7 , 28.9 ± 1.6 , and 109.8 ± 6.6 on day 352 (December 18) (Fig 2). There were no differences in size between males and females in any treatments in September (day 269), but males were significantly larger than females in treatments 5 and 6 in December (day 352). Maturing males were significantly larger than nonmaturing males and females at the end of the experiment (Figure 3). A large drop in condition factor occurred in September, suggesting that the fish smolted at this time (Figure 4). Overall mortality totaled 33 fish and ranged from 1 to 5 fish per tank. Mortality was not attributable to any single cause. Water quality parameters remained within acceptable levels for salmonids (Wedemeyer 1996).

Body fat levels were related to ration level, although there was a strong seasonal trend. Fat levels in all groups increased from first feeding to early fall, remained relatively constant during the fall and winter, then declined during the spring and summer (Figure 5). Body fat levels in treatment 6 fish were significantly higher than fish in the other groups from day 269 onward. At the end of the experiment in July, maturing males in all treatments had higher levels of body fat than nonmaturing males and females, which had similar levels of body fat (Figure 6).

A total of 2,900 fish were examined at the end of the experiment in July (day 562). There were 1,377 females, 1,071 nonmaturing males, and 452 maturing males. ANOVA indicated significant differences in the incidence of maturing males among treatments (Table 1). Regression of tank mean weights in December (the approximate time of initiation) and the incidence of male maturation using a quadratic equation provided a good fit to this relationship ($r^2 = 0.938$; $y = -0.010x^2 + 1.633x - 1.869$; Figure 7). As the fish in all treatments maintained their relative size difference throughout the experiment, this relationship would have been similar for each month of the experiment.

Data (male weight on December 1 vs. percent maturation in July) from the current experiment and data from two previous experiments conducted with this stock in our laboratory (Silverstein et al. 1998, Shearer et al. 2000) were fitted with a quadratic relationship in a meta-analysis. The values from the study by Silverstein et al. (1998) were 36.4, 31.6, 21.3, and 7% maturation for fish that were 30, 27, 15, and 13 g. The values from Shearer et al. (2000) were 81.9, 87.5, 92.8, 88.0, 75.8, 66.2 and 78.9% for fish that were estimated to be 119, 100, 92, 79, 64, 51 and 79 g. Fish weights on December 1 were estimated using regression (quadratic) analysis with November and December sample weights from each experiment. This analysis (Figure 8) suggests that the average threshold size below which maturation at age 1⁺ would not occur for this stock, under our rearing conditions, is 7.9 g ($y = -0.015x^2 + 2.418x - 14.545$, $r^2 = 0.923$).

Body fat appears to influence the incidence of maturation in smaller fish. This can be seen if data on body fat in December, and male maturation rates, are plotted for the four experiments conducted at our laboratory since 1993 (Shearer and Swanson 2000, Silverstein et al. 1998, Shearer et al. 2000). Large fish (>50 g in December) matured at a high incidence even with low levels of body fat. In small fish (<50 g in December), there appears to be a relatively strong effect of fat on maturation (Figure 9).

The distribution of plasma 11-KT levels in December 2000 showed a bimodal split in values for each treatment. The percentages of fish falling into the upper mode for treatment 2, 4, and 6 were 17%, 20%, and 42%, respectively (Figure 10). Plasma 11-KT level was the only parameter measured that showed a bimodal split in proportions reflecting the final rate of maturation eventually observed for these three treatment groups. Histological examination of the testis for all groups did not show the presence of any late type-B spermatogonia, thus maturing and immature fish could not be separated based on this criterion. When fish were divided into mature and immature groups, based on the bimodal distribution of plasma 11-KT levels, maturing males (upper KT mode) in treatment 6 had significantly higher body weight ($p < 0.05$) than nonmaturing males, and maturing males in treatment 6 and 4 had significantly higher GSIs than nonmaturing males ($p < 0.05$). All other parameters measured did not significantly differ between maturing and nonmaturing males.

Average plasma IGF-I and 11-KT levels increased significantly as body weight increased among the three treatments. Highest levels occurred in fish in treatment 6 while the lowest levels occurred in treatment 2 (Table 2). A similar trend was found with GSI data, but differences were only significant between treatments 6 and 2. Fish in treatments 2 and 4 had similar average whole body fat levels, whereas fish in treatment 6 had significantly higher whole body fat than fish in the other two treatments. When the data from all three groups were combined, plasma IGF-I levels were positively correlated with size (Figure 11, $r^2 = 0.856$).

Discussion

Previous work (Shearer and Swanson 2000, Silverstein et al. 1998, Shearer et al. 2000), together with that of others (Hopkins and Unwin 1997, Clarke and Blackburn 1994) has shown that growth rate and, to a lesser extent, fatness in smaller fish appear to be major biotic factors affecting the incidence of early male maturation in chinook salmon. Therefore, the objective of our experiment was to determine if there is a threshold body size below which there would be no early sexual maturation in male spring chinook salmon.

The growth rates produced in the current study were generally lower than those produced in earlier studies with this stock (Silverstein et al. 1998, Shearer et al. 2000). In the current study, fish in the smallest group were approximately 10 g in December, while in the largest group they were approximately 108 g. At a similar time of year, wild and hatchery fish of this stock are about 8-9 g and >50 g, respectively (J. Leppink and J. Ziller, Oregon Department of Fish and Wildlife, pers. commun.). Although maturing

males were significantly larger than nonmaturing males at the end of the experiment, the differences were small with the exception of treatment 6. This was most likely due to ration restriction in treatments 1-5.

Body fat levels were similar to fish fed the same feed in an earlier experiment (Shearer et al. 2000) up to day 200, the fish fed the restricted rations (treatments 1-5) showed little change until the following spring. Fish fed at a higher level continued to increase in body fat. In the spring prior to maturation, fish in treatments 1-5 declined significantly in body fat. If the decline in whole body fat observed in fish in treatments 1-5 had continued this might have compromised fish health. The low fat levels could have been due to ration restriction and the relatively warm temperatures during the spring and summer (about 11°C). Fish in treatment 1 were being fed less than 0.5% of body weight/day. Higher body fat levels in the maturing compared to nonmaturing males and females, have been observed in our previous experiments (Silverstein et al. 1998, Shearer et al. 2000, Shearer and Swanson 2000).

In an earlier experiment (Silverstein et al. 1998), a 7% maturation rate was observed in fish that had an average weight of 13 g in December. With this exception, the maturation rates we observed in the smaller fish in the present study were much lower than we have reported previously (Silverstein et al. 1998, Shearer et al. 2000, Shearer and Swanson 2000). Based on the meta-analysis, this appears to be due primarily to fish size. However, we cannot pinpoint the exact size or time when size or growth rate is 'assessed' by the fish because in all of these studies relative differences in fish size were present over the course of experiments. Although our results confirm that fish size, or a correlate to size, is the most important determinant of whether a 1+ age male will sexually mature, the relationship between body fat levels and the incidence of maturation also reinforces our earlier observation that body fat plays a role in smaller fish.

By mid-December, histological analyses of the testes in each group could not distinguish between maturing and nonmaturing males based on the appearance of late (secondary) type-B spermatogonia. Maturation in males, or spermatogenesis, consists of the initial mitotic proliferation of stem cells and spermatogonia, differentiation of primary to secondary-B spermatogonia followed by meiosis and differentiation of spermatids into spermatotozoa (see Schulz 2000, Loir 1999). The classical histological determination of initiation of spermatogenesis is based on the appearance of the secondary B spermatogonia that occurs just prior to initiation of the first meiotic division (Schultz 2000). However, several mitotic and differentiative steps precede the secondary B spermatogonia stage and constitute earlier stages of spermatogenesis. Unfortunately, these early types of spermatogonia are difficult to identify consistently by histology. However, there is accumulating evidence for the role of 11-KT in the stimulation of these earlier mitotic stages (Miura et al. 1991, Nader et al. 1999), and it has been shown that plasma 11-KT levels in December are good indicators of maturation in the following year (Shearer et al. 2000).

Although a relatively small number of males were sampled (<30) per group in the present study, it was still possible to see a bimodal distribution in 11-KT plasma levels.

The proportion of individuals in the upper mode of plasma 11-KT levels reflected the final rates of maturation in the three groups tested. None of the other parameters measured showed a consistent bimodal separation throughout the three groups. It is hypothesized that the elevated plasma 11-KT observed in the fish in the upper mode in December indicates an activation of the reproductive endocrine axis and onset of puberty. It is likely that the 11-KT is involved in the stimulation of the early mitotic proliferation of type-A and early type-B spermatogonia, and differentiation of early-B to late-B spermatogonia.

For each of the three groups sampled in December, there was a trend for the males designated as “maturing” by 11 KT levels to be larger than immature males, but this only became significant for the fish in the high ration treatment 6 where the range of body weights sampled was highest. We expect that a similar relationship would have been observed in the other groups if the distributions in size were greater. Plasma levels of IGF-I were positively correlated with fish size, as previously reported (Beckman et al. 2001, Pierce et al. in press). In addition, IGF-I has been shown to have stimulatory effects on the early mitotic stages of spermatogenesis in fish (Loir and Le Gac 1994, Loir 1999b, Nader et al. 1999) and it has been hypothesized that this may be a mechanism whereby body growth regulates the initiation of maturation. In the present study, although higher plasma IGF-I tended to be associated with higher maturation rate, body size also showed a similar trend. Therefore, the relationship between plasma IGF-I levels and maturation, remains unclear.

With the exception of the work by Brett and coworkers (Brett et al. 1969, Brett 1979, Brett and Groves 1979, Brett et al. 1982, Brett 1995) there is relatively little published on the bioenergetics of juvenile Pacific salmon. It is clear, however, that growth, fatness, and early male sexual maturation are affected by the interaction among diet composition, ration level, and temperature in juvenile male chinook salmon. One of our earlier studies (Shearer et al. 1997) demonstrated that body composition and growth could be independently manipulated. But in that study the effect of temperature was not taken into account. In contrast, Brett et al. (1969) examined the effect of temperature and ration size on growth and body composition but did not examine the role of diet composition. Brett (1979) made the point that conducting and interpreting multifactorial bioenergetics experiments are difficult. The results of our experiment suggest that it may be difficult to grow fish slowly at warmer temperatures because the whole body fat levels in the fish fed at the lowest ration level appeared to be reaching a critically low level at the end of the experiment. Further understanding of more complex interactions (e.g., temperature x fish size x ration size x diet composition) and development of rearing strategies to prevent early male maturation will require further experimentation. Finally, size or growth-rate threshold for initiation of maturation may differ among stocks; however, results of this study indicate that, in spring chinook salmon, the incidence of male maturation of 1+ age fish can be significantly reduced by reducing growth in the first year of rearing. One critical question remaining is when during the season growth can be accelerated without causing maturation.

Acknowledgements

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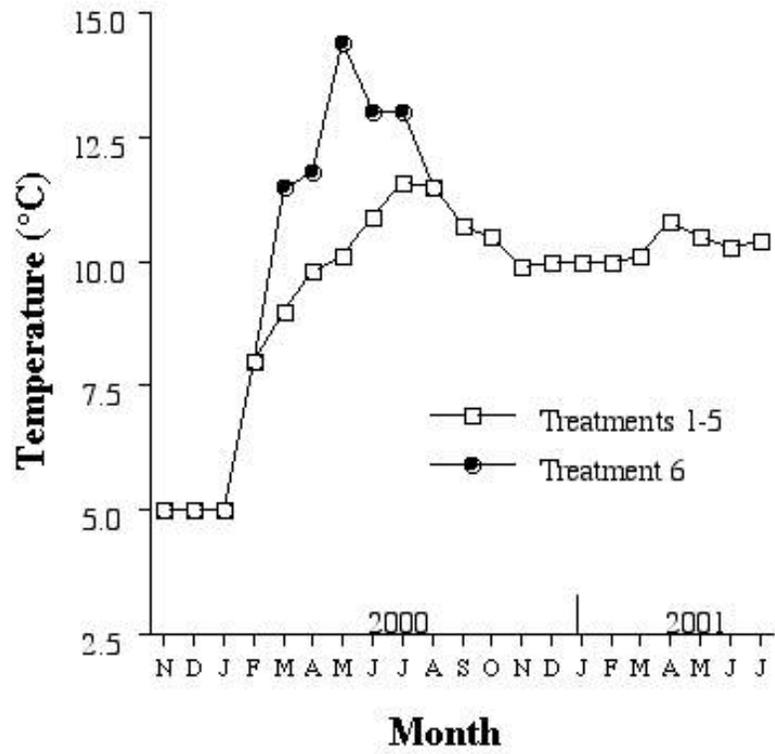


Figure 1. Mean monthly temperature over the course of the experiment.

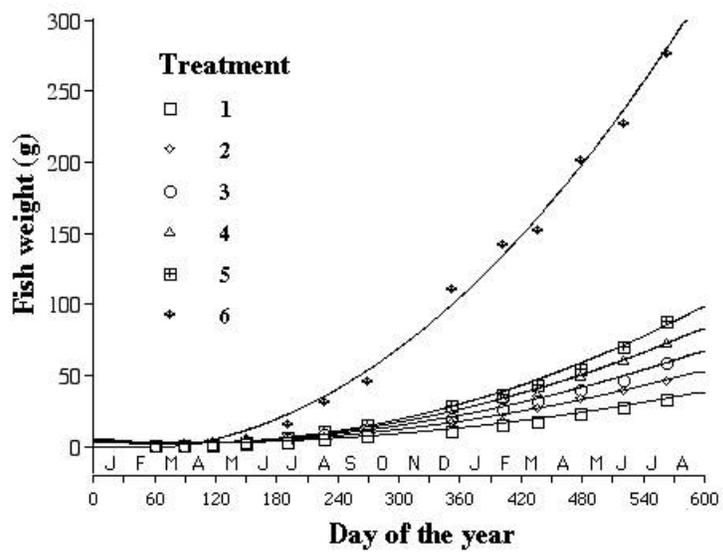


Figure 2. Fish weight (g) over the course of the experiment. Each point is the mean of two tanks.

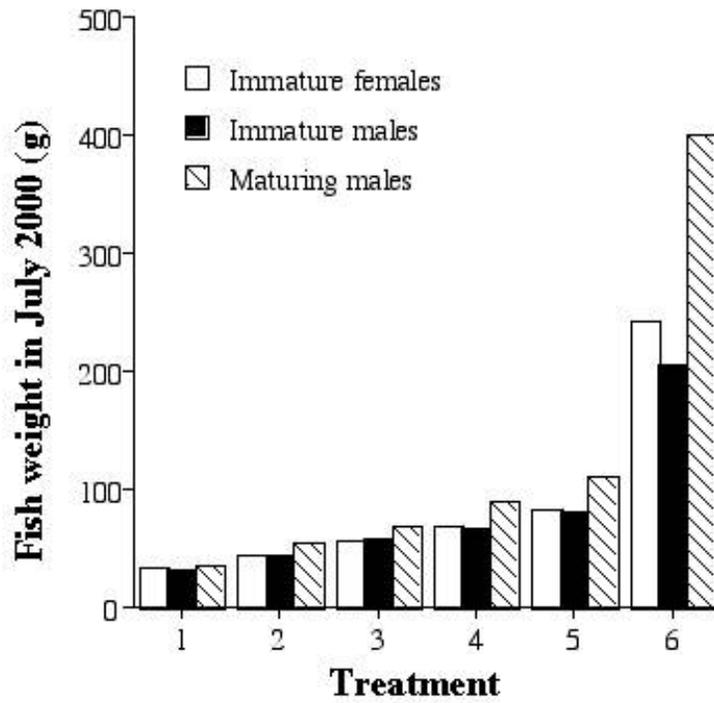


Figure 3. Mean weights of females, nonmaturing males, and maturing males at the end of the experiment in July.

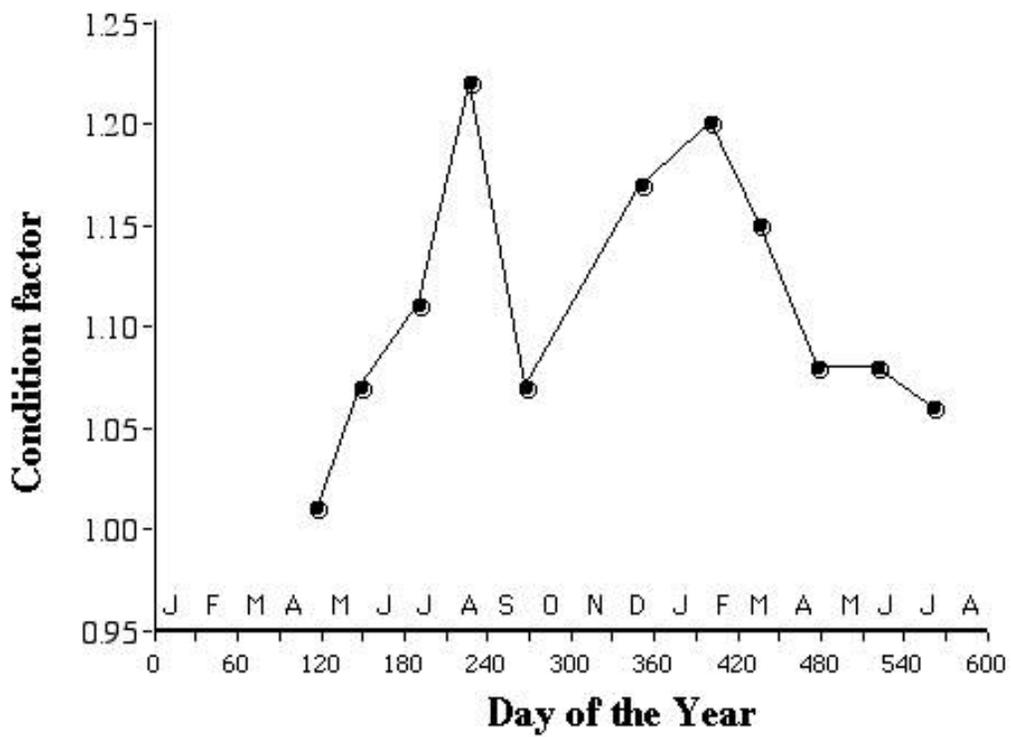


Figure 4. Condition factor over the course of the experiment (all treatments combined).

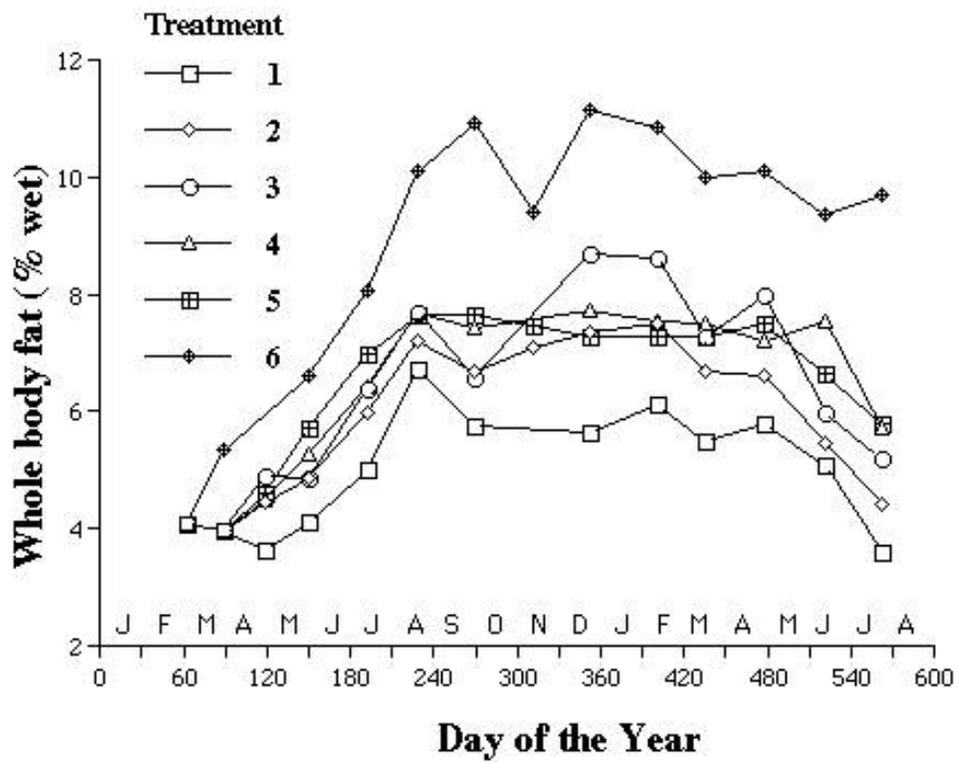


Figure 5. Whole body fat levels in fish fed graded levels of a commercial feed.

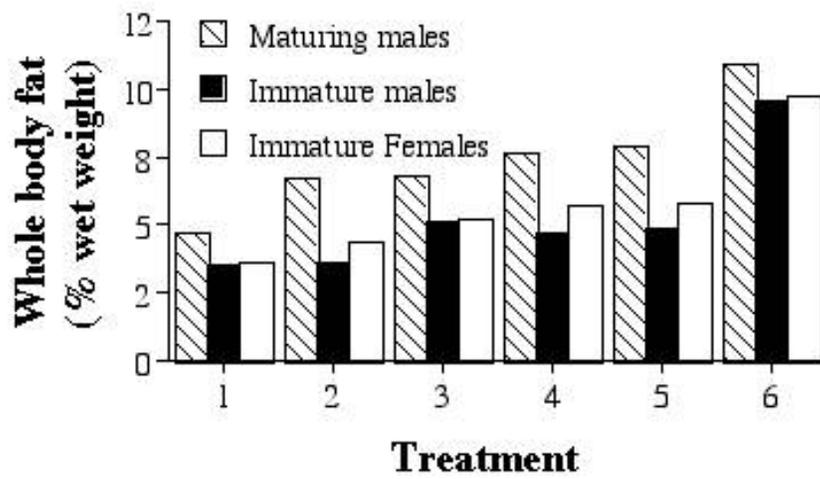


Figure 6. Whole body fat levels at the end of the experiment in July.

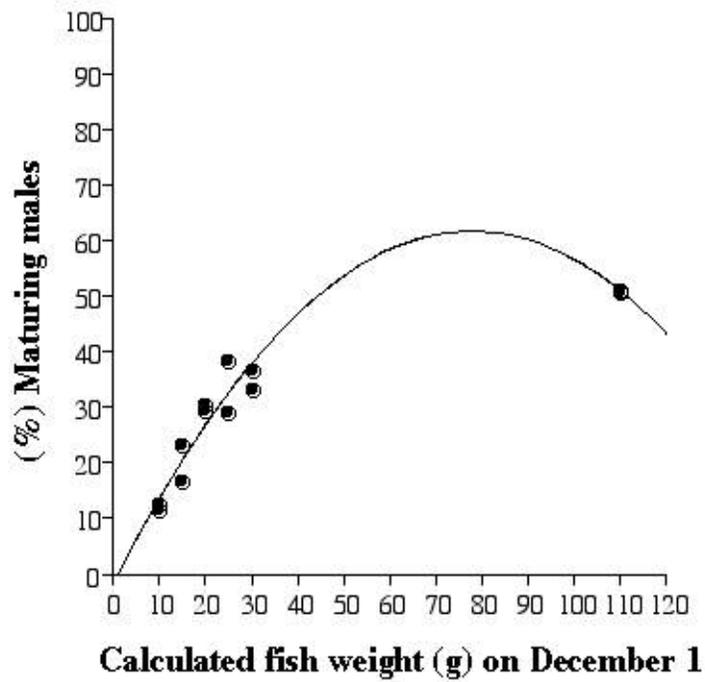


Figure 7. Mean fish weight in each tank in December versus the incidence of maturing males in July. Data points represent individual tanks ($Y = -0.010x^2 + 1.633x - 1.869$, $r^2 = 0.938$).

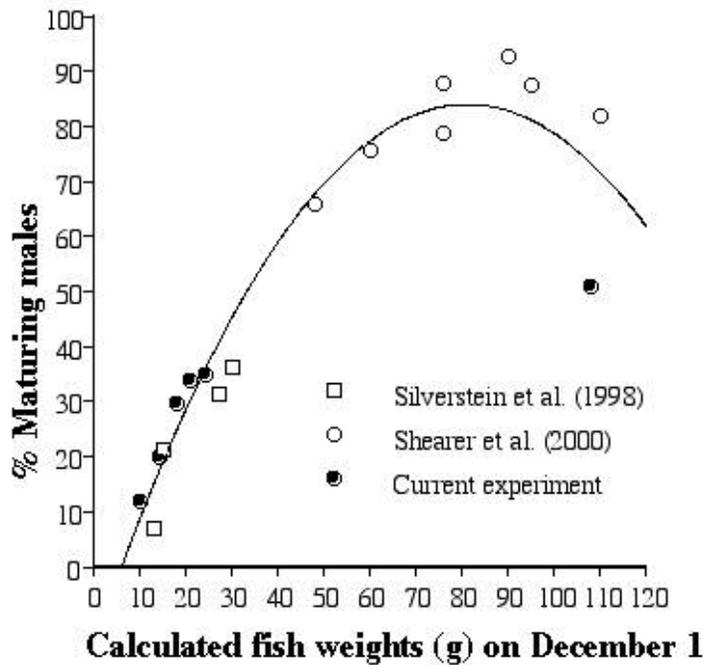


Figure 8. Mean fish weights in December versus the incidence of maturing males in July. Each data point is the mean of two replicate tanks. Data from the current experiment, Shearer et al. (2000) and Silverstein et al. (1998). A quadratic equation ($y = -0.015x^2 + 2.418X - 14.545$, $r^2 = 0.923$) predicts that the threshold size for maturation to occur is less than 7.9 g.

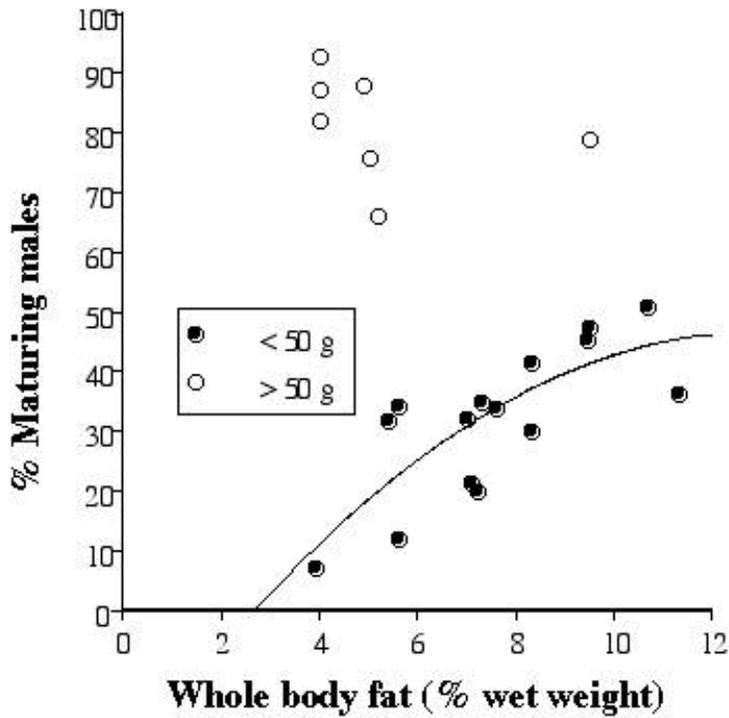


Figure 9. The relationship between whole body fat in December and the incidence of early male sexual maturation. The data are from Shearer and Swanson (2000), Silverstein et al. (1998), Shearer et al. (2000) and the present study. The open circles are from Shearer et al. (2000) and were very large lean fish. In larger fish it appears that the effect of fat level is of little significance. For the smaller fish (filled circles), $y = -0.437x^2 + 11.405x - 27.906$, $r^2 = 0.605$.

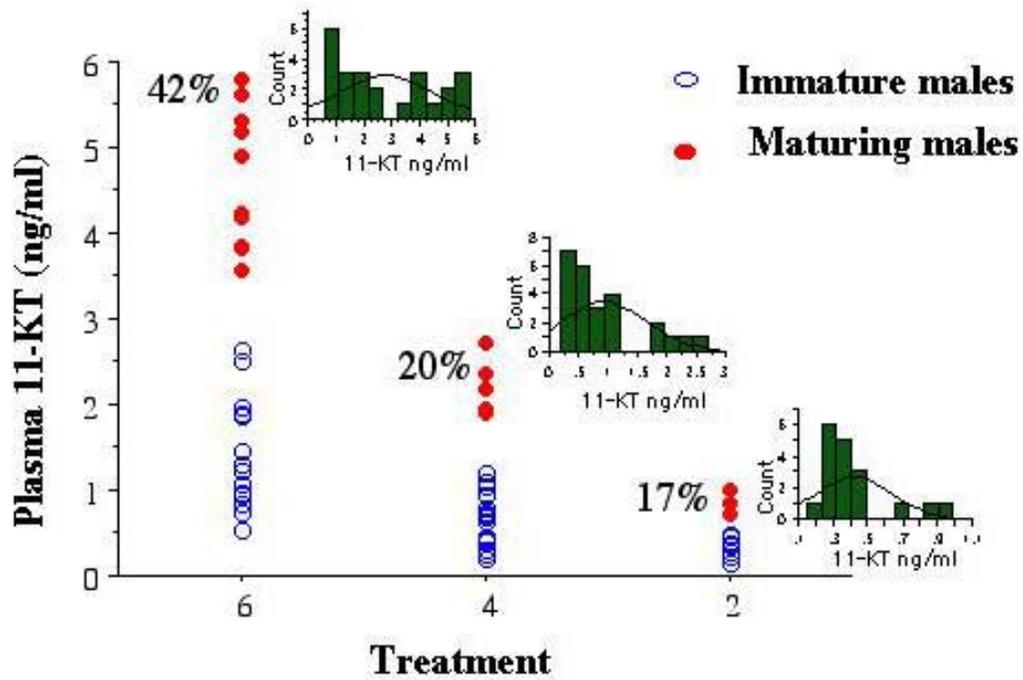


Figure 10. Distribution of plasma 11-KT (ng/ml) within the three treatments. Percentages indicate the number of predicted maturing fish determined by distribution for each treatment (6, 4 and 2) group. Inset histograms show frequency distributions for each group.

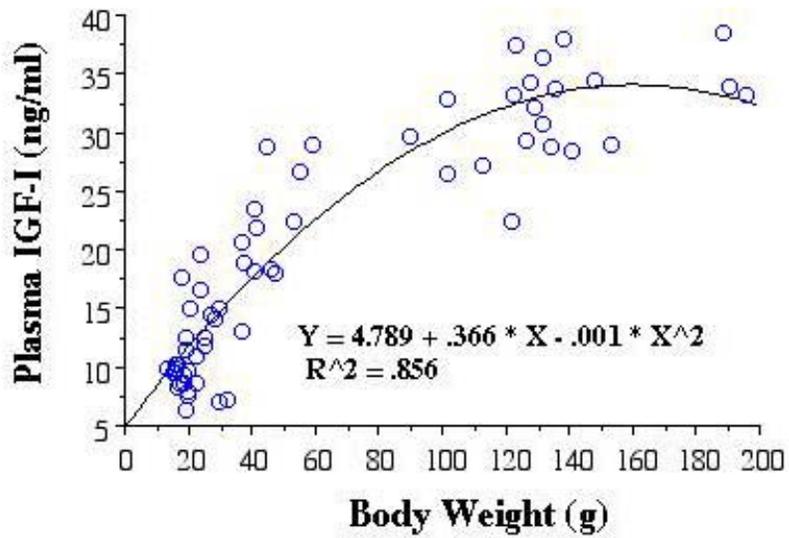


Figure 11. Relationship between body weight and plasma IGF-I levels for the three experimental treatments (6, 4 and 2) combined. Data points represent individual fish. $y = -0.001x^2 + .366x + 4.789$, $r^2 = 0.856$.

Table 1. Percent maturing males in July, GSI in July, and male weight in December

Treatment	Male maturation ¹ July (%)	GSI of maturing males July (%)	Actual male weight on 18 December (g)	Estimated male weight on 1 December (g) ²
1	12.0±0.5 ^a	5.03±0.38	10.9±1.3	10.1
2	19.9±3.4 ^a	6.21±0.35	16.4±0.4	14.0
3	29.9±0.6 ^b	7.16±0.36	19.3±0.8	17.9
4	33.8±4.7 ^b	6.97±0.25	22.1±1.7	21.0
5	34.9±1.6 ^b	7.25±0.24	28.9±1.6	24.0
6	51.0±0.2 ^c	6.47±0.18	110.0±6.6	108
P	<0001	0.70		

¹Values with different superscripts are significantly different based on ANOVA.

²Estimated weight on 1 December based on extrapolation between sample weights in November and January using a quadratic regression.

**TASK 5. EFFECTS OF GROWTH ON AGE OF MATURATION, FECUNDITY
AND EGG SIZE IN FEMALE COHO SALMON**

(FINAL REPORT: 1 JUNE, 2001 THROUGH 31 MAY, 2002)

by

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Introduction

One of the major aims of captive broodstock programs for recovery of depleted stocks of Pacific salmon is to optimize the production of fertile gametes from adult fish that will either be introduced to the wild or spawned artificially in captivity. However, various forms of reproductive dysfunction have been observed which limit the desired production from captively-reared fish, and drastically reduce the effectiveness of such programs for recovery of depleted stocks.

Both the Redfish Lake sockeye salmon and Snake River spring chinook salmon captive broodstock programs have encountered problems with highly variable egg quality (range: 0-90%, and averages of 40-70% survival to eyed-stage). This is much lower than that of populations of anadromous fish spawned artificially on return to the hatchery. Captively-reared females also display a high rate of egg retention and, in some cases, abnormal ovarian development leading to reduced egg size and number.

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 (see Thorpe 1998, Duston and Saunders 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). For example, because of the variable nature of the incubation and early rearing environment in the freshwater habitat, it has been hypothesized that a type of adaptive phenotypic plasticity occurs, whereby some aspect

of juvenile rearing influences egg size to optimize offspring survival. 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). It is unclear how, and precisely when, adjustments in reproductive investments (egg number, egg size) are made, and whether they coincide with the proposed decision periods for onset of maturation.

Clearly growth history has a pivotal role in controlling not only the initiation and maintenance of the process of sexual maturation in salmonids, but also influences other aspects of reproductive performance. In an artificial rearing environment it is possible that abnormal ovarian development may result from inappropriate growth during critical periods when life history decisions on maturation initiation and adjustments in reproductive investment are being made. One of the goals of captive broodstock programs has been to produce adult fish comparable in size to fish from the wild population of the same stock. There has been a focus on feeding high quality broodstock diets during the year of expected maturity. Far less consideration is given to the effect of juvenile rearing strategies on the subsequent reproductive performance of adults 1-3 years later. In semelparous species, determining the timing and aspects of growth which influence the physiological commitment of oocytes to mature is crucial, as there is no second opportunity once this commitment is made.

In the present study, growth in coho salmon was manipulated during their adult seawater-rearing phase (smolt to adult) in order to determine critical periods when growth affects ovarian development. Coho salmon were selected for this study because of the simple life history and the availability of fish from a previous study that examined the effects of growth rate on smoltification.

Methods

The experimental approach was to produce female coho salmon of a range of body sizes and growth rates by varying ration and to determine the relationship between body growth during various points of the life cycle and ovarian development. Yearling coho salmon (1997 brood) were obtained from the Minter Creek Hatchery, WA and reared in 12-ft diameter fiberglass tanks supplied with filtered and UV-treated seawater at the Manchester Research Station (MRS). Fish were individually tagged with PIT tags prior to transfer to seawater as smolts during April 1999. Fish (1,300) were divided into two groups, low growth and high growth. Using the growth model based on the delta-I method of Piper (1982), a daily ration was calculated to produce fish of 210 g and 280 g (low and high growth respectively) by 26 February 2000. 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 (Moore Clark, BroodSelect). Periodic sampling for growth, body composition, and plasma hormone levels were made regularly during 1999

as part of another study on smoltification (B. Beckman, unpublished). In October 1999, 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. Because of limited tank space and seawater, most of the males were culled from the experiment.

In January 2000, fish were sorted by size into two groups; those above the median size (large) and those below the median (small). These two groups were subdivided into another two groups with different projected target sizes for 3 May 2000. The new high growth and low growth groups were projected to reach 320 g and 225 g body weight by May, respectively. Thus, there were four treatments (70 females, 10 males, one tank per treatment): big fish high growth, big fish low growth, small fish high growth, and small fish low growth. The rearranging of fish between the two target growth regimes in February was done to ensure a range of growth histories that were not correlated with the initial size of the fish in April 1999. Subsequent to 3 May 2000, all groups were fed to apparent satiation until 6 November 2000 when food was withdrawn and the maturing fish were transferred to freshwater tanks. From 6 November through 27 December 2000, fish were checked every 4-7 days for ovulation. Water temperature declined from 9°C to 2.5°C during this rearing period. Immature females remained in seawater and were fed a maintenance ration until termination in January 2000.

To monitor growth during the adult rearing period, body weight and length were recorded bimonthly for all fish from January 2000 through July 2000, and during October 2000. At spawning, data on body weight, body length, total ovary mass, fecundity, average egg weight, and spawning date were recorded. For females that did not mature at 3 years of age in December 2000, data on body length and weight, and ovarian weight were recorded in January 2001. All fish were killed at this point and samples of ovaries of the nonmaturing fish were fixed in Bouin's fixative for 24 hr and then stored in 70% ethanol for subsequent histological analysis.

Mortality was a major problem during this experiment, with approximately 53% of the females dying of an unidentified tail rot syndrome prior to the spawning period in November 2000. Most of these mortalities occurred in the spring of 2000, and all females were treated with feed containing florfenicol (15 mg/kg body wt./day for 14 days) on two occasions during this period. Females were also treated with an intraperitoneal injection of florfenicol (30mg/kg body wt.) during May 2000. These mortalities precluded any of the proposed periodic sampling we had planned in order to correlate changes in the reproductive endocrine axis with growth history.

Spawning data (body weight, body length, total ovary mass, fecundity and average egg weight) were also obtained from adult Minter Creek Hatchery (adipose fin-clipped) and wild (non-adipose fin-clipped) fish returning to Minter Creek after a year of ocean rearing. Hatchery fish had been raised in the hatchery and released as smolts; therefore, these fish had the same early freshwater growth history as the fish reared in this experiment at Manchester, followed by growth in seawater in the ocean. Wild fish were

from the same Minter Creek stock, but both early freshwater and seawater growth occurred in the wild (Figure 1).

Comparisons of size and growth rates among the three developmental groups held at Manchester (see below) were made using ANOVA. The relationships between egg parameters and length were analyzed by simple regression. The effect of the three different rearing environments (Manchester, Hatchery, and Wild) on the relationship between egg parameters (ovary mass, fecundity and egg size) and body size were determined by comparisons of regression slopes using ANCOVA. The relationships between final reproductive parameters and growth history were analyzed by multiple step-wise regressions. For regression and ANCOVA analyses, data were log transformed in order to conform to test criteria. All analyses were performed using Staviw (Abacus Inc., CA).

Results

Growth and maturation of experimental fish reared at Manchester

At age 3, 60% (79 out of 131) of the females matured and were spawned during November and December 2000. The remaining 40% (52 out of 131) of the females did not mature at age 3. When gonad weight in the nonmaturing fish in January 2001 was regressed against body weight, two clear groups appeared (Figure 2). A strong positive relationship between body size and gonad weight ($r^2 = 0.648$, $P < 0.0001$, $n = 23$) was found in fish in the first group that had gonadosomatic indices (GSIs) over 0.4% (Figure 3). A weak, but significant relationship between body size and gonad weight ($r^2 = 0.249$, $P < 0.01$, $n = 29$) was found in a second group of fish that had GSIs less than 0.4% (Figure 3). Histological analyses (data not shown) indicated that the ovaries of fish with a GSI less than 0.4% contained many atretic oocytes; whereas, fish with a GSI greater than 0.4% had normal appearing oocytes in early stages of vitellogenesis. Therefore, all data on growth for the Manchester reared fish were split into three developmental groups: maturing females (spawned Fall 2000), nonmaturing females with large developing ovaries (NM-LO), and nonmaturing females with small, abnormal ovaries (NM-SO).

A peak in specific growth rate occurred in all three groups during the first summer in seawater with a decline in the fall and winter (Figures 4 and 5). Subsequently, the specific growth rate of maturing females increased from May to July, and decreased between July and September prior to spawning in December. A similar trend was found in immature fish, but with lower specific growth rates than maturing fish.

Significant differences in the pattern of changes in growth rate and body size were observed among the three groups (Figures 4 and 5). Initially in April 1999, maturing females were significantly smaller than nonmaturing fish with normal ovaries; however, maturing females were significantly larger than the nonmaturing fish with normal ovaries from November 1999 onwards. This was due reduced growth rate from June 1999 onwards in the nonmaturing fish with normal ovaries.

Body weights in the nonmaturing fish with abnormal ovaries were similar to maturing fish from April 1999 through February 2000; however, from May 2000 through the end of the experiment, body weights of the nonmaturing fish with abnormal ovaries were significantly lower than fish that matured in December 2000. The lower body size observed for the nonmaturing fish with abnormal ovaries in May 2000 resulted from a significantly lower growth rate in this group from November 1999 onward compared with maturing females.

By January 2001, there was no difference in average final body weight between the two groups of nonmaturing fish; however, the pattern of growth over time in these two groups differed considerably. Initially, nonmaturing fish with normal ovaries were larger than the nonmaturing fish with abnormal ovaries in April 1999. However, the nonmaturing fish with abnormal ovaries had higher growth rate from June 1999 through November 1999 compared with the nonmaturing fish with normal ovaries. Growth rate between November and December 1999 in the nonmaturing fish with abnormal ovaries fell to a level similar to that of the nonmaturing fish with normal ovaries. Subsequently, these two groups had similar growth rate and size through January 2001, except between July and October 2000 when nonmaturing fish with abnormal ovaries had significantly lower growth rate than fish with normal ovaries.

Rearing history and egg size versus egg number

Females (Wild or Hatchery) returning to the Minter Creek Hatchery were over twice the weight of those females reared in seawater at Manchester (Table 1) and had significantly higher total ovary mass, fecundity, and egg weight. Wild and Hatchery females were similar in body size, total ovary mass, and fecundity, but Wild females had significantly higher egg weight than Hatchery females (Table 1). Although females reared at Manchester had, on average, smaller eggs than fish from the other two rearing environments, they also had the individual smallest and largest eggs of the three groups (see minimum and maximum values for egg weight, Table 1 and Figure 7).

For fish in all three rearing environments (Manchester, Hatchery, and Wild), significant positive relationships were found between body size (length or stripped weight) and total ovary mass (Figure 5), fecundity (Figure 6), and egg weight (Figure 7). Length explained over 80% of the variation in ovary mass for fish from both the Hatchery and Manchester rearing environments, and 67% of the variation in ovary mass in Wild fish (Table 2). Length explained over 60% of the variation in fecundity for fish from both the Hatchery and Manchester rearing environments. A weaker relationship between length and fecundity was found in Wild fish ($r^2 = 0.53$). Length explained 44.9% and 33.7% of the variation in egg weight for fish from the Hatchery and Wild rearing environments, respectively. The weakest relationship between length and egg weight was found for the females reared at Manchester ($r^2 = 0.21$). No significant differences were found for the slopes of the regression analyses of data from the three rearing environments (ANCOVA $P > 0.05$), indicating that a change in body size produced a similar change in ovary mass, fecundity, and egg weight, irrespective of rearing histories.

Specific growth history and egg size versus egg number

Growth history and final body size--In the Manchester-reared fish, simple linear regression analyses of final strip body weight or length at spawning versus body weight or length at each sampling period (Table 3) indicated that the strongest relationship existed in the summer months just prior to spawning (May 2000 $r^2 = 0.49$ and July 2000 $r^2 = 0.83$). This suggests that final body size in maturing females is primarily influenced by growth during the summer prior to spawning. A relatively weak relationship ($r^2 = 0.19-0.23$) was found between final body size and size during the first fall and winter in seawater (August 1999 - February 2000). No relationship between final size and growth during smoltification in April 1999 ($r^2 = 0.009$) was evident. However, this is not surprising because the experimental design mixed fish between the high and low ration in the winter to ensure a range of growth histories that were not necessarily correlated with the body size at the outset of the experiment.

Growth history and reproductive parameters--Using simple linear regression analyses of total ovary mass versus body length or weight at each sampling period, a similar relationship to that of final body size was found. The strongest correlation (i.e. largest r^2) occurred in the summer months preceding spawning (Table 4). There was a significant correlation between fecundity and final body size, but the r^2 value was lower than that of the regression of total ovary mass and final body size. Working back in time, this relationship was strongest during the summer months preceding spawning and gradually weakened until no significant relationship between size and fecundity was detected at smoltification. A relatively weak relationship was found between egg weight and final strip body weight, the relationship was even weaker during the summer before spawning.

Egg size and fecundity trade-off--To examine the relationship between egg size and egg number, the residuals of \log_{10} fecundity versus \log_{10} length were plotted against \log_{10} egg size versus \log_{10} length (Figure 8). A significant negative relationship was found between these residuals for fish from the Manchester and Hatchery rearing environments ($r^2 = 0.496$ and 0.364 , respectively, $P < 0.001$). No significant relationship ($r^2 = 0.041$, $P > 0.05$) was found for these residuals for fish from the Wild rearing environment. Thus, fish from the Manchester and Hatchery rearing environments that had larger eggs than explained by body size alone also had fewer eggs than explained by body size alone. Similarly, fish that had smaller eggs than explained by body size alone also had more eggs than explained by body size alone. A multiple regression analysis was performed to examine the relationship between growth parameters and final egg size. In this analysis only body size in October and final body size entered into the equation significantly. None of the growth parameters prior to October contributed to the multiple regression model for the final egg size.

Discussion

Growth and maturation

Maturing compared with nonmaturing females--The majority of female coho salmon of the Minter Creek stock normally spawn at three years of age; however, in this experiment a significant proportion of the three-year old fish reared at Manchester did not mature. Among the females reared in this experiment at Manchester, the maturing females had higher growth than nonmaturing females from the spring as 1+ age fish (smolting) onwards. However, maturing females returning to the Minter Creek hatchery were two and an half times larger than the same age maturing fish reared at Manchester. This suggests that, although growth in seawater for some of the Manchester-reared fish was sufficient to support maturation, it was substantially lower than that of anadromous wild or hatchery fish of the same stock and year class.

Previous work, in 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 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).

The difference in size and growth rate between the maturing and nonmaturing fish reared at Manchester in the present study supports these theories, but cannot provide additional information to the timing of such decision periods as growth was different between these groups from April 1999 through the end of the experiment. However, examination of the growth histories of the two types of nonmaturing fish that were observed in this study lends support for a period of "initiation of maturation" in the fall and a "permissive period" in the late winter and spring.

When the nonmaturing fish were sacrificed in January 2001, two types of fish were observed. One type had large ovaries with immature oocytes and the other type had small ovaries with atretic oocytes. The fish with atretic oocytes had similar growth to the maturing fish during the fall "initiation" period, but significantly lower growth than maturing fish during the late winter/spring "permissive" period. Presumably, these fish initiated secondary oocyte growth but, because of reduced growth in the following winter and spring, maturation was inhibited and atresia ensued. On the other hand, nonmaturing fish with large ovaries and immature oocytes had significantly lower growth than maturing fish in the fall "initiation" period, suggesting that maturation was not initiated and oocytes remained in an immature state. Although the physiological cause of ovarian

atresia in this group of fish is unclear, the fact that this developmentally distinct group of females displayed unique growth patterns during the two proposed critical periods of maturation decisions supports the idea that maturation is in a state of flux at such times.

Among the maturing females of the Minter Creek stock a large range of size at maturation was observed. The relatively small body size of maturing females reared at Manchester indicates that the genetically set thresholds for the initiation and continuation of maturation in this stock are likely to be relatively low. The majority of the fish in the wild would likely surpass these thresholds, and return and spawn as 3 year-old fish. It is possible that females that do not surpass these thresholds in the wild are too small to survive to maturity and therefore, do not return to the hatchery. The large difference in spawning size between the females returning to the hatchery and those at Manchester is assumed to be due to poor growth in the spring, subsequent to the second maturational decision period.

Nonmaturing females--Female coho salmon that failed to mature at three years of age at Manchester would generally be expected to mature the following fall at age 4. However, when these fish were sacrificed they appeared to fall into two distinct groups based on GSI and appearance of the ovary. One group, with small ovaries containing healthy immature oocytes had $GSI > 0.4$. The other group had small ovaries with atretic oocytes and a $GSI < 0.4$. While it is possible that females with small healthy ovaries would mature at age 4, it is unlikely that the females with atretic oocytes would mature at any age. Atresia is a process of degeneration of oocytes and provides a mechanism by which the final number of ovulated eggs can be adjusted during periods of ovarian growth (Bromage and Cumaratunga 1988, Tyler and Sumpter 1996). An increase in the number of atretic oocytes has been associated with reduced ration and growth (Springate et al. 1985, Bromage and Cumaratunga 1988, Bromage and Jones 1991, Bromage et al. 1991). It is likely that the ovarian atresia observed in the 3+ age females in January 2001 was due to reduced growth from June to October 2000. The impacts of massive ovarian atresia at an early stage of oogenesis could be substantial for semelparous salmonids because it is unclear whether primitive germ cells (stem cells or primary oogonia) remain and can be recruited into secondary oocyte growth the subsequent year. Unlike iteroparous females, such as rainbow trout, a reserve of primary oocytes is not present in the ovary once vitellogenesis ensues in semelparous salmonids. In iteroparous fish, the primary oogonia constitute the pool from which the eggs will develop in subsequent years (Chestnut 1970, Ishida et al. 1961). Thus, in a semelparous salmon, atresia may permanently reduce the number of developing oocytes, and if the rate of atresia is high enough too few oocytes would survive to develop and the female would likely become sterile. Indeed, this has been observed in captive broodstock programs for Redfish Lake sockeye salmon where some females of age 5 and 6 years did not exhibit secondary sex characteristics and, when examined, the ovaries appeared to be composed of connective tissue and small atretic eggs (K. Johnson, IDFG, pers. commun.).

We hypothesize that growth during critical periods not only affects the physiological commitment to initiate secondary oocyte growth and maturation but also

regulates adjustment of oocyte number via the processes of atresia. In extreme cases of severe growth reduction, the ovary may undergo a very high rate of atresia resulting in a large reduction in fecundity or even sterility. This is supported by the finding that, although the growth of the maturing females at Manchester was sufficient to permit maturation, these fish showed a reduced fecundity compared with the much larger females returning to the hatchery. Furthermore, widespread ovarian atresia was apparent in nonmaturing fish which appeared to grow poorly during the “permissive” spring period.

Rearing environment and egg production.

For all three rearing environments, a significant positive relationship was found between ovary mass, egg number and egg size and final body size. Egg size has been identified as an important reproductive trait that can contribute significantly to the fitness of an individual (e.g. Hutchings 1991, Einum and Fleming 2000). Thus, for an endangered species recovery program, obtaining final body sizes approaching those of wild returning females is important not only to increase the number of eggs produced but also to provide appropriate egg size. This is particularly important for captive broodstock programs that introduce fertilized eggs back into the natural habitat using egg boxes, or that release captively-reared females to spawn in their native streams.

Of the three reproductive parameters measured (ovary mass, egg number and egg size) the weakest relationship was consistently observed between egg size and final body size for all three rearing environments. Indeed, some individuals raised at Manchester produced eggs of a similar size to fish from the other two rearing environments that were up to 3-4 times heavier at spawning. Thus, although egg size is significantly influenced by final body size there are other factors which significantly contribute to this reproductive trait. Jonsson et al. (1996) and Morita et al. (1999) have correlated a low growth rate in early freshwater stages with the subsequent production of larger but fewer eggs. In the present study, if egg size was related to growth during the freshwater rearing period, one would expect differences to appear between the fish initially raised in the stream (Wild) and those reared in the hatchery (Hatchery and Manchester), as wild outmigrants are typically smaller than hatchery reared fish (Salo and Bayliff, 1958). Wild females produced significantly larger eggs than females from both the Hatchery and Manchester rearing environments. However, Hatchery fish also produced significantly bigger eggs than the fish grown at Manchester. Thus, no consistent correlation between the early freshwater rearing and egg size was found in this study. In addition, for females grown at Manchester, final body size explained 21% of the variance observed in egg weight and no other growth parameter, including size at the end of the juvenile freshwater rearing period (April 1999), significantly improved the regression model. No evidence was found to indicate that size at the end of the freshwater growth stage influenced final egg size.

For the two groups that were raised in the same hatchery during their early freshwater stage (Manchester and Hatchery) there was a direct trade-off between egg size and fecundity when the effect of body size was removed. In other words, as egg size

increased, egg number decreased in the hatchery-reared fish. No such relationship was observed for wild fish reared in the natural stream environment (Wild). For the Wild females the relationships between body size and fecundity, and body size and ovary mass were weaker than those found for the other two rearing environments. Thus, there was more variation in the relationship between body size and reproductive traits for females initially grown in a stream environment compared with those raised in the hatchery (Manchester and Hatchery). It remains unclear how this difference in the variation between body size and reproductive traits originates, and what significance it has on the quantity and quality of gametes produced. It is possible that the uniform rearing environment in the early life history stages reduces variation, and this variation itself may be adaptive for the population.

Conclusions

- For Minter Creek coho salmon, a reduced size and growth rate from smolting onwards resulted in a failure to mature at 3 years of age.
- Maturation was not initiated in fish that had poor growth during the first fall in seawater. Widespread atresia of oocytes occurred in fish that had good growth in the fall, but reduced growth in the late winter and spring. Ovarian atresia, may result in the production of individuals with reduced fecundity or, in extreme cases, sterility. Examination of ovaries with severe atresia revealed no primary oocytes, suggesting that the fish would be sterile.
- Ovary mass and fecundity were highly correlated to final body size, and the final body size was largely produced by the rapid increase in growth during the spring and summer just prior to spawning in the fall.
- Final body size was significantly related to final egg size indicating that attaining a 'wild type' body size at spawning is important for the production of a 'wild type' egg size.
- A large proportion of the variation in egg size was not explained by final body size, and none of the growth parameters from smoltification onwards provided a further explanation of egg size.
- Egg size was largely influenced by a direct trade-off with egg number.
- We detected no consistent effect of early freshwater rearing environment on subsequent egg size, although there was an apparent difference in the variation between body size and reproductive traits.

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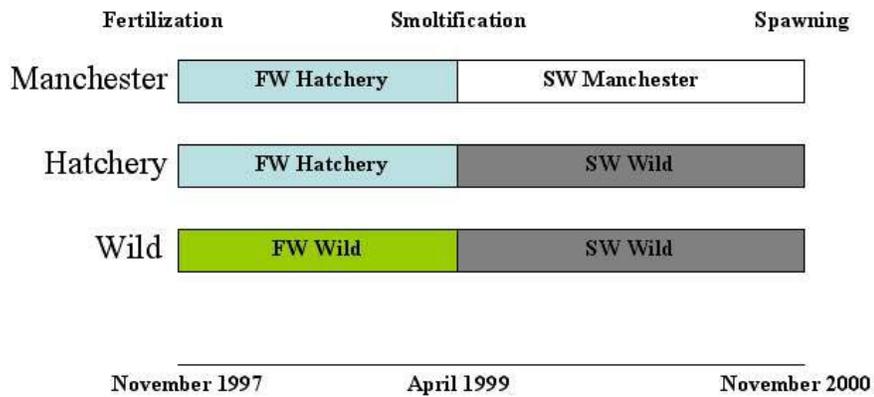


Figure 1. Time line for the three rearing environments for coho salmon reared at Manchester Research Station (Manchester), Minter Creek Hatchery (Hatchery), and wild fish sampled in Minter Creek (Wild).

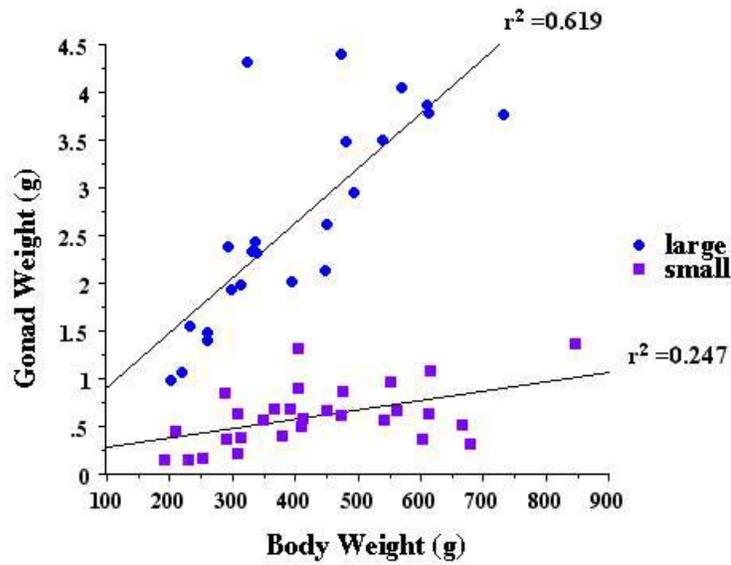


Figure 2. Regression analysis of body weight and gonad weight from the nonmaturing female coho salmon sampled in January 2001. Large = fish with GSI >0.4%, Small = fish with GSI <0.4 %. See Figure 3.

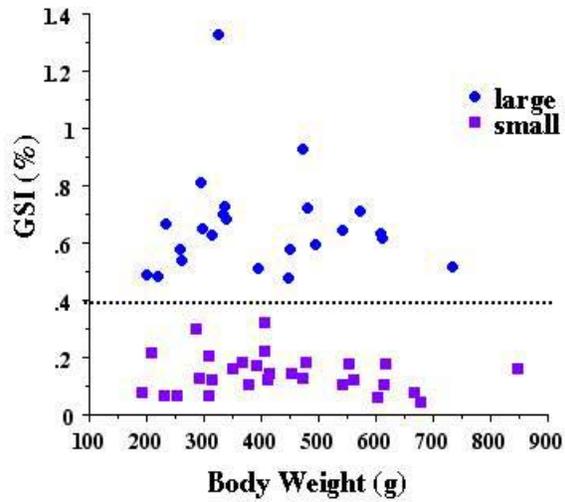


Figure 3. Scatter plot of body weight and gonadosomatic index (GSI) for the nonmaturing female coho salmon sampled in January 2001. Dashed line indicates the cut off point designating large (GSI >0.4%) or small (GSI <0.4%) groups.

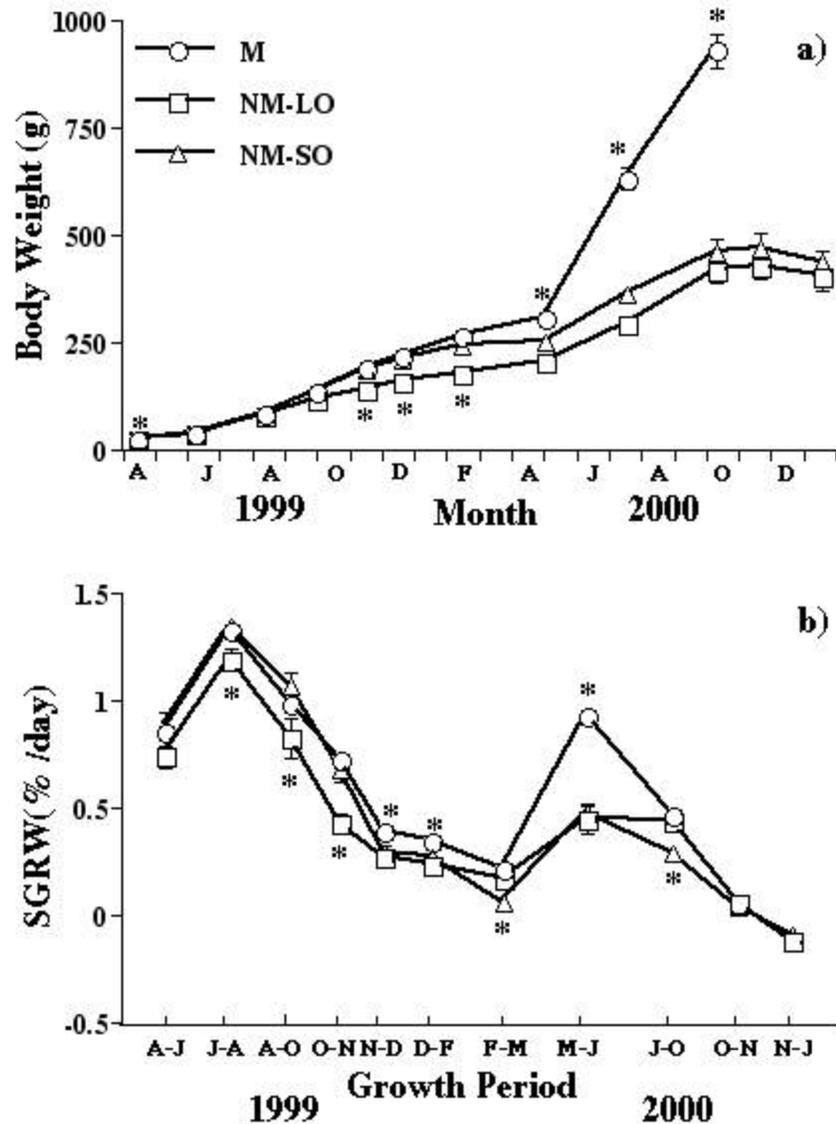


Figure 4. Mean body weights (top panel) and specific growth rate (bottom panel) by body weight of the three developmental groups during the rearing period: maturing (M), nonmaturing with large developing ovaries (NM-LO), and nonmaturing with small, abnormal ovaries (NM-SO). *indicates significantly different from the other two groups at that time point.

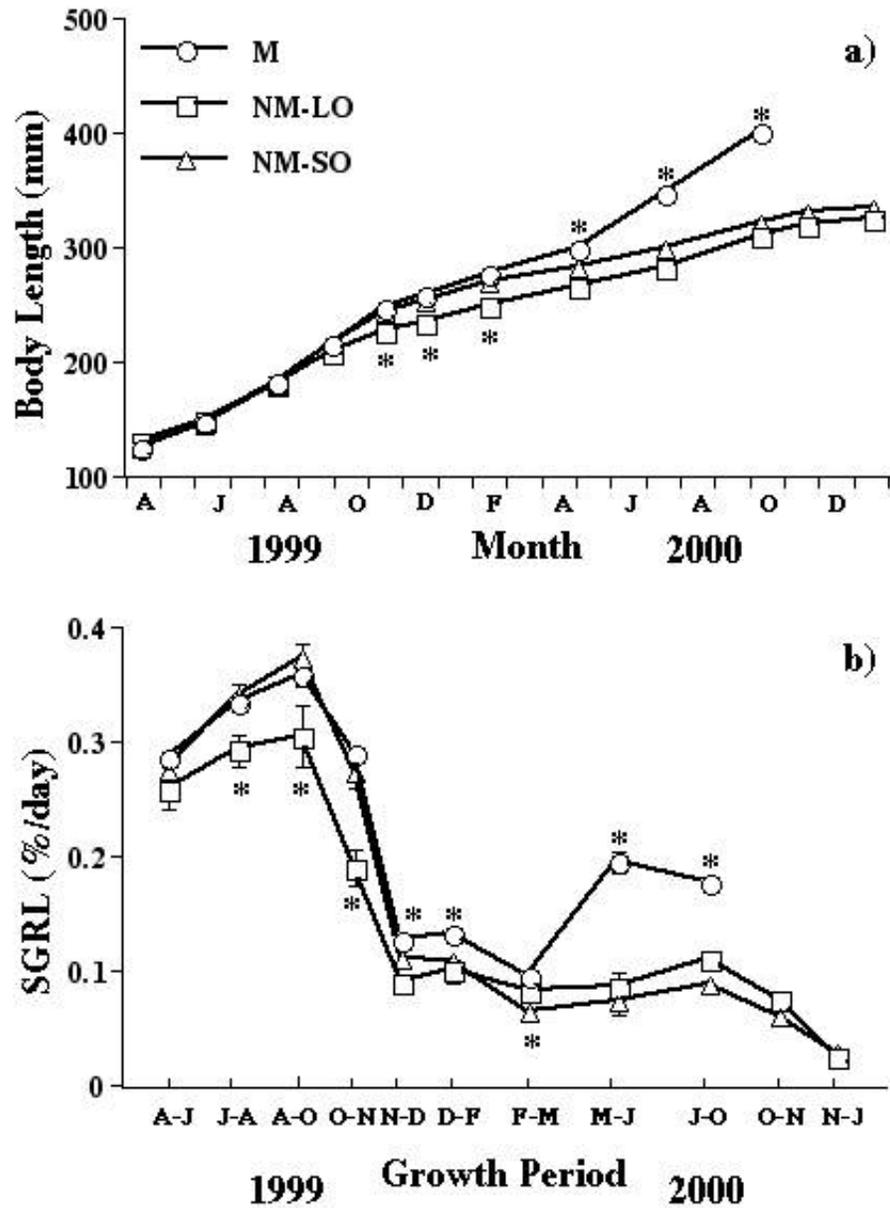


Figure 5. Mean body length (top panel) and specific growth rate (bottom panel) by body length of the three developmental groups during the rearing period: maturing (M), nonmaturing with large developing ovaries (NM-LO), and nonmaturing with small, abnormal ovaries (NM-SO). *indicates significantly different from the other two groups at that time point.

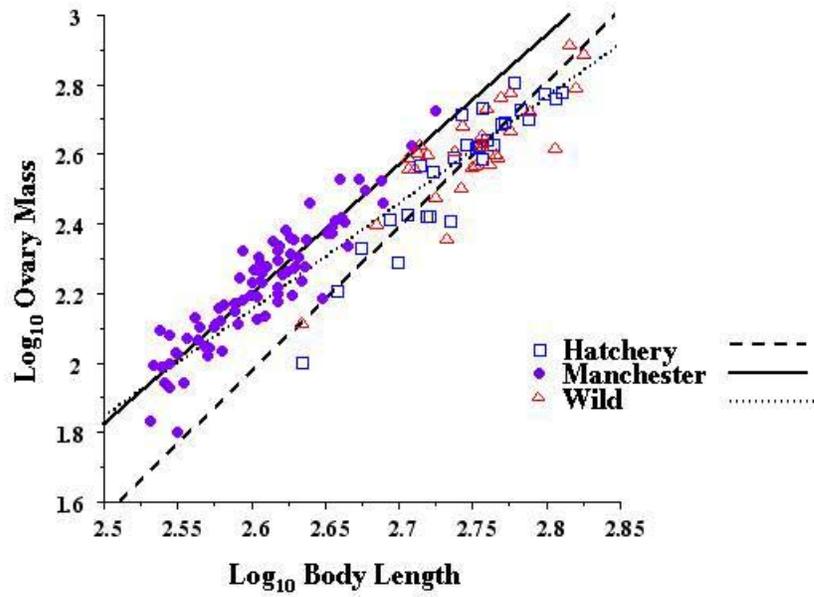


Figure 6. Regression analysis of Log₁₀ total ovary mass and Log₁₀ body length at spawning for coho salmon from the three rearing environments; Manchester, Hatchery, and Wild (see Figure 1).

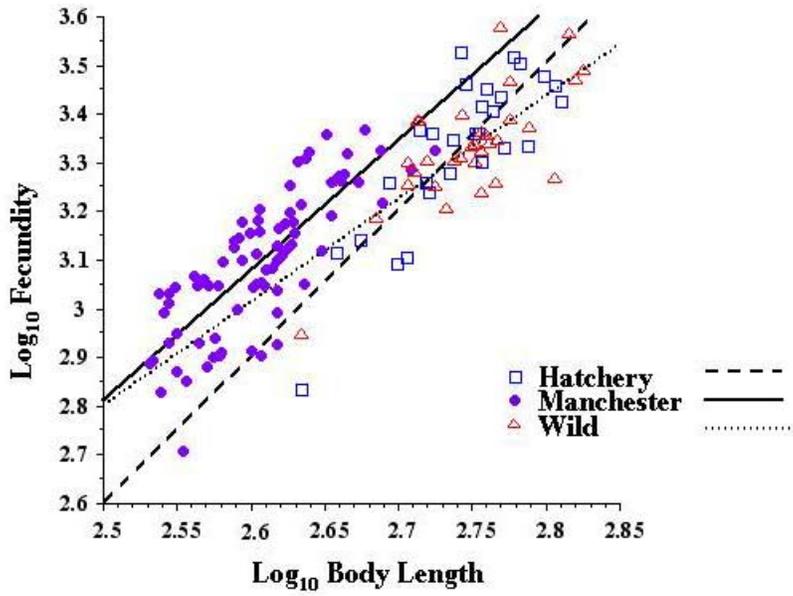


Figure 7. Regression analysis of Log₁₀ total fecundity and Log₁₀ body length at spawning for coho salmon from the three rearing environments; Manchester, Hatchery, and Wild.

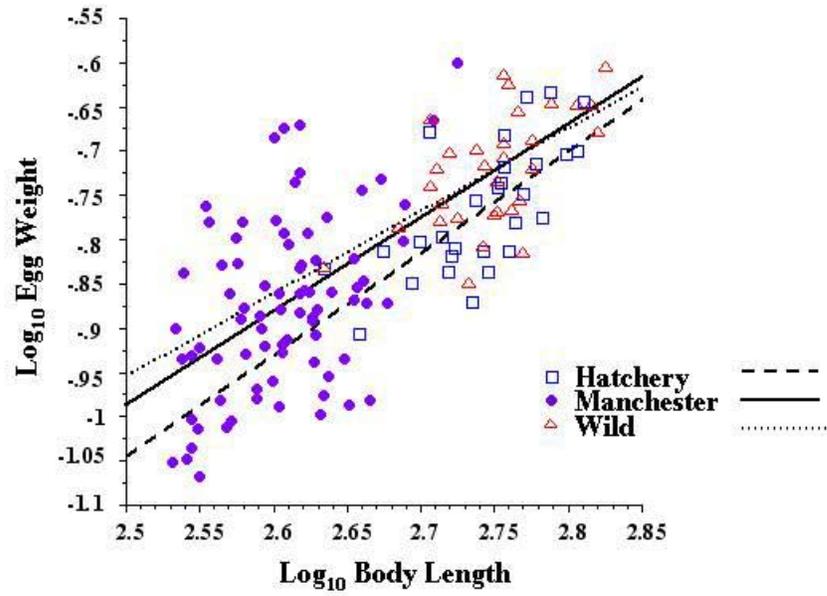


Figure 8. Regression analysis of Log₁₀ egg weight and Log₁₀ body length at spawning for coho salmon from the three rearing environments; Manchester, Hatchery, and Wild.

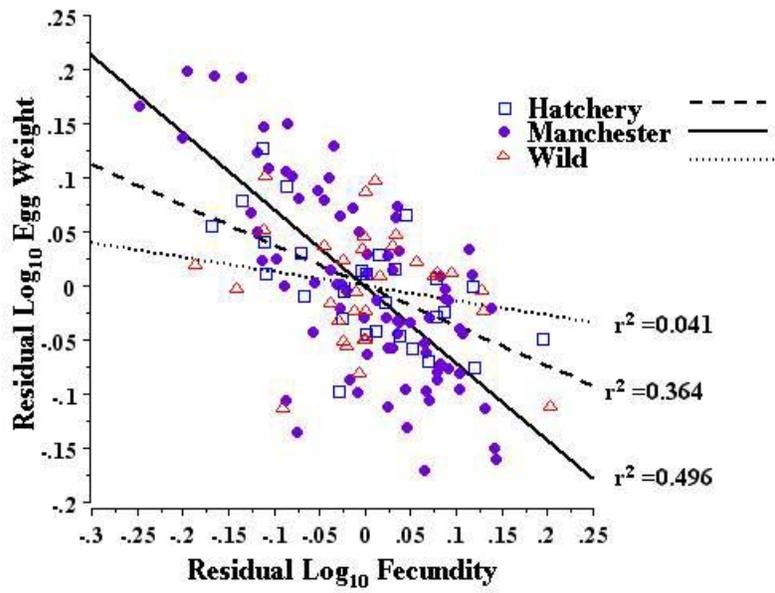


Figure 9. Regression analysis of the residuals from Log₁₀ egg weight versus Log₁₀ body length and Log₁₀ fecundity versus of Log₁₀ body length for fish from the three rearing environments; Manchester, Hatchery, and Wild.

Table 1. Strip body weight (g), body length (mm), ovary mass (g), fecundity (n), and egg weight (g) for the three rearing environments (Manchester, Hatchery, and Wild). Numbers represent mean \pm S.E. Within each parameter, superscripts indicate statistically significant differences ($P < 0.05$).

Rearing Environment	n	Strip Weight (g)	Length (mm)	Ovary Mass (g)	Fecundity (#)	Egg Weight (g) (min-max)
Manchester	79	642 \pm 25 ^a	407 \pm 5 ^a	182 \pm 10 ^a	1321 \pm 48 ^a	0.138 \pm 0.004 ^a (0.085-0.251)
Hatchery	28	1547 \pm 87 ^b	553 \pm 10 ^b	398 \pm 28 ^b	2251 \pm 129 ^b	0.174 \pm 0.006 ^b (0.124-0.233)
Wild	31	1676 \pm 92 ^b	561 \pm 9 ^b	429 \pm 25 ^b	2219 \pm 106 ^b	0.192 \pm 0.005 ^c (0.107-0.248)

Table 2. Regression equations and analyses of Log₁₀Ovary Mass (LOM), Log₁₀Fecundity (LF), or Log₁₀Egg Weight (LEWt) on Log₁₀Length (LL) for the three rearing environments, Hatchery, Manchester, and Wild.

Parameter	Rearing Environment	Slope+Intercept ^a	r ²	df	F	P
Log ₁₀ Ovary Mass (LOM)	Hatchery	LOM=-8.866+4.17LL	.873	27	179.4	<0.0001
	Manchester	LOM=-7.496+3.728LL	.852	78	441.9	<0.0001
	Wild	LOM=-5.781+3.052LL	.670	30	58.8	<0.0001
Log ₁₀ Fecundity (LF)	Hatchery	LF=-4.948+3.02LL	.698	27	60.1	<0.0001
	Manchester	LF=-3.873+2.673LL	.637	78	135.4	<0.0001
	Wild	LF=-2.489+2.117LL	.535	30	33.3	<0.0001
Log ₁₀ Egg Weight (LEWt)	Hatchery	LEWt=-3.917+1.15LL	.469	27	22.9	<0.0001
	Manchester	LEWt=-3.623+1.055LL	.212	78	20.7	<0.0001
	Wild	LEWt=-3.29+0.934LL	.337	30	14.7	0.0006

^a Slopes between rearing environments with each parameter are not significantly different by ANCOVA (P >0.05)

Table 3. Regression coefficients (r^2) for the relationship between stripped body weight (at spawning in December 2000) and length or weight at a given date.

Month	weight	length
October 2000	0.98	0.92
July 2000	0.83	0.79
May 2000	0.49	0.43
February 2000	0.22	0.21
December 1999	0.23	0.18
November 1999	0.2	0.17
September 1999	0.22	0.23
August 1999	0.19	0.17
June 1999	0.07	0.05
April 1999	0.009	0.008

Table 4. Regression coefficients (r^2) for the relationship between weight at a given date and total egg mass, individual egg size, and fecundity at spawning. Fish spawned in December 2000

Month	Total egg mass (g)	Individual egg size (g)	Fecundity
October 2000	0.94	0.27	0.66
July 2000	0.81	0.18	0.64
May 2000	0.50	0.13	0.33
February 2000	0.20	0.06	0.10
December 1999	0.20	0.06	0.10
November 1999	0.16	0.05	0.08
September 1999	0.19	0.07	0.07
August 1999	0.18	ns	0.12
June 1999	0.05	ns	0.05
April 1999	ns	ns	Ns

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.

(PROGRESS REPORT: 1 JUNE 2000 THROUGH 31 MAY 2002)

by

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INTRODUCTION

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. The delay in spawning time in captively reared fish relative to wild fish of the same stock is not uncommon. Other captive broodstock programs for Oregon stocks of Snake River spring chinook salmon, Redfish Lake sockeye salmon and Sacramento River winter run chinook salmon have observed varying degrees of delayed spawning. The degree of the spawning delay varies with the stock and even year to year within the same stock of fish. 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 early in the 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 are likely due to rearing environment since overt genetic selection for spawn time has not occurred in the captive broodstock programs. 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. Abnormal ovarian development may be due to rearing conditions 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 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.

Therefore, in the present study, reproductive development was compared in two stocks of Snake River spring chinook salmon: adults returning to the IDFG Rapid River Hatchery and Lemhi River captive broodstock. The goals of the study were to compare the progress of sexual maturation by histological analyses of gonad samples and measurement of reproductive hormones, to evaluate the bioenergetics of migration in the migrating hatchery fish, and to 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 hormonal changes, 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 captive broodstock are far less than that of wild fish that undergo an upriver migration. The large returns of adult chinook during 2001 provided a unique opportunity to obtain important physiological information from migrating adult spring chinook salmon. The data will provide a template by which to compare captively-reared adult chinook salmon, and important information to aid in understanding the underlying causes of the delayed maturation in the captive broodstock.

Work Completed

Materials and Methods

Sampling protocol- Samples of gonads, blood, pituitary glands and carcasses were collected from adults that returned to the IDFG Rapid River Fish Hatchery (Riggins, Idaho) at monthly intervals from May through spawning in September 2001. A total of 10 males and 10 females were sacrificed each month. Photographs using a digital camera were taken of whole bodies and gonads. 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), August (prior to the normal time of release into the spawning stream) and September/October (when fish were spawned in the hatchery). Six to nine females at each time point were sampled. Lemhi River captive broodstock were reared in filtered and UV-treated saltwater at the NMFS Manchester Research Station (MRS) from May 1999 through early June 2001. On June 8, 2001 fish were transferred to fresh water and reared through spawning at the IDFG Eagle Hatchery, Eagle, ID. In June 2001, an additional treatment group was added to determine if temperature of fresh water influenced spawn timing. Lemhi River captive broodstock were divided into two groups: chilled (9 °C) and ambient (14 °C). Samples were collected from females in both treatments during August and at spawning.

During the sampling of Lemhi River captive broodstock in May, 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, 10 females from the Catherine Creek spring chinook salmon captive broodstock maintained in fresh water at the Bonneville Hatchery, and in saltwater at the MRS were sampled during May.

Histology- Testicular tissue was 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. 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.

Plasma and pituitary hormone analyses- Blood was collected from caudal vein using 18 gauge needles and heparinized syringes. Blood was immediately transferred to 15 ml polypropylene tubes containing aprotinin and trypsin inhibitor and stored on ice prior to centrifugation. Plasma was stored on dry ice until transferred to -70 °C freezer for long term storage. Pituitary glands were frozen immediately on dry ice and stored at -70 °C until analyzed 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 17 β (E) were determined in plasma samples by either RIAs or enzyme immunoassays (EIA) (Cuisset et al. 1994, Rodriguez et al. 2001, Sower and Schreck 1982). Plasma insulin-like growth factor (IGF-I) was measured using GroPep components as described by Shimizu et al. (1999).

Body composition- Carcasses, gonads and visceral 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 subsample of 100 g of wet material was dried, then reground in a coffee grinder, and a subsample was taken for analysis (0.5 g for protein and 2 g 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.

Morphometrics- Fish were euthanized and photographed with a digital camera on their left side along side a ruler. Cartesian coordinate information for 19 landmarks was collected from each image with the digitizing program *tpsDig* (Rohlf 1998a). Thirty-two distance measurements were calculated between 15 landmarks in a truss network pattern 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 1998b) 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.

Work to be Completed

As of May 2002, analyses of hormone levels in plasma and pituitary samples were completed with the exception of plasma vitellogenin (females) and 17 β , 20 β -dihydroxy-4-pregnen-3-one (both sexes). It is anticipated that these will be completed by December 2002. Analyses of carcass, viscera, and gonad composition are completed and a preliminary analysis of the data has been completed. Initial analyses of morphometric data are completed. Histological analyses of testis development in male Rapid River spring chinook salmon are completed, but ovarian histology has not yet been completed. Samples have been processed, but quantification of oocyte stages by image analysis will be done during the FY02 work period. Data on spawning time have been collected and analyzed. All data collection and analyses will be completed during the FY02 work period and will be reported in the final report for FY02.

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**TASK 9. DETERMINE CRITICAL IMPRINTING PERIODS FOR SOCKEYE
SALMON**

(PROGRESS REPORT: 1 JUNE, 2001 THROUGH 31 MAY, 2002)

by

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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 to within a very close proximity of their natal habitat. Captive rearing programs often occur at locations that do not provide the same water source that the fish would experience in its natural habitat, and therefore, the timing of releases 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 that illustrates 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 NMFS in 1991 and in that same year the 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 that 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 salmon into the wild that includes stocking of eyed eggs, net pen and direct lake releases of pre-smolts, smolt releases, and releasing captively-reared adults to spawn naturally. Some of the fish for these releases were reared at the Sawtooth Hatchery, however, the majority of fish are reared at several out-of-basin facilities (NMFS hatcheries at Manchester and Big Beef Creek, Washington; IDFG hatchery at Eagle, Idaho, and ODFW Bonneville hatchery) because there were no

appropriate Stanley Basin facilities, 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.

The spread-the-risk strategy for reintroduction of Redfish Lake sockeye salmon is necessitated in part by our 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) salmon 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 that are 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 that will maximize survival without increasing straying.

Approach

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 rear 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 2002 and 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 summed 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.

Work completed

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 immediately migrate to a lake, where they remain for 1 or 2 years before smolting and migrating to sea. To determine the relative importance of odorant exposure during these key developmental periods and the importance of exposure duration,

sockeye salmon were exposed to specific odorants as alevins/emergent fry (the period just prior to and during emergence from the natal gravel) (February, 2001) or were exposed as smolts (April-May, 2002). Specifically, 4,000 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 also have a relative extensive migration downstream and upstream migration through the Columbia Basin and hydroelectric facilities. Embryos were transferred to the NWFSC and reared in chilled dechlorinated Seattle City water. Fish were divided into three treatment groups: 1) alevins/emergent fry exposure, 2) smolt exposure, 3) control. The smolt exposure group will be further divided into three 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 1 February to 5 March, 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 the University of Washington's Big Beef Creek Research Station in August 2001 and will be maintained there until the end of the experiment. From February 2002 until 1 June 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. 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 (15 April – 24 May, 2002); one week (29 April – 24 May, 2002); or 1 day (24 May, 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), and releases of smolts into outlet streams (1 day). All groups were maintained separately until after the parr-smolt transformation (31 May, 2002), then marked by treatment and will be reared communally to maturity.

Assessment of imprinting in sockeye will begin in fall 2002 by measuring olfactory sensitivity to exposure odorants using electro-olfactograms (EOG), a relatively simple electrophysiological technique that has been 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. Briefly, the EOG technique involves placing a recording electrode near the medial surface of one lamella within the olfactory rosette and monitoring electrical activity in response to perfusion of the rosette with odorants. 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, and preliminary results suggest that salmon exposed to amino acid odorants during sensitive imprinting periods demonstrated a heightened sensitivity to these odorants relative to control fish. EOG assessment and validation with coho salmon will continue through fiscal year 2002-2003.

Work to be completed

During fall 2002, 10 fish/treatment group will be tested for heightened EOG sensitivity to the exposure odors relative to reference odorants. Differences between treatment groups in EOG responsiveness to specific odorants will be examined by analysis of variance (ANOVA) followed by Fisher's PLSD. Significance for all analyses will be established at the $P < 0.05$ level. We are also collecting and freezing olfactory rosettes from all sacrificed fish for later analysis of odorant receptor mRNA levels. This molecular approach to assay for olfactory imprinting is being developed as part of another project but may be easily adapted to this study if successful.

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 (C. McAuley, NMFS, pers. commun.) 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, we will test the efficacy of GnRH analog implants for inducing EOG olfactory sensitivity to imprinting odorants in odorant-exposed fish. If successful, GnRH implants will also be used for EOG evaluations of odorant-exposed sockeye in fall 2002 to determine the timing of imprinting in the experimental sockeye salmon.

Behavioral testing of odorant-exposed salmon will begin 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.

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**TASK 10. USE OF ANTIBIOTICS AND NEW VACCINES TO REDUCE
MORTALITY FROM BACTERIAL KIDNEY DISEASE IN CHINOOK SALMON**

(FINAL REPORT: 1 JUNE 2001 THROUGH 31 MAY 2002)

(Experiment A. Study the efficacy of other vaccines)

by

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Introduction

Bacterial kidney disease (BKD) is the major infectious disease preventing successful culture of salmonids in the Pacific Northwest. BKD-caused epizootics continue to significantly impact captive rearing programs of both sockeye and chinook salmon (Schiewe et al. 1997). There is no vaccine available to protect salmon from infections with *Renibacterium salmoninarum*, the causative bacterium 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 extensive efforts to reduce the incidence of disease with long-term prophylactic administration of the antibiotic erythromycin, culling of eggs based on BKD-enzyme-linked immunosorbent assay (ELISA) values, and vaccination with commercial vaccines (unproven in Pacific salmonids). With respect to erythromycin, new data suggest that repeated doses have a deleterious effect on gamete viability and other measures of reproductive success (see progress report for “Evaluation of Toxic Effects of Long-term Prophylactic Use of Azithromycin and Erythromycin” under this same Task). 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 will continue by necessity.

In general, long-term prophylactic use of antibiotics should be avoided where possible. In order to reduce antibiotic treatment of BKD, 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, or bacterins, heat-treated killed whole cells (to reduce the amount of p57 protein on the surface, a putative major virulence factor), and purified p57 (Piganelli et al. 1999).

Recently, Aqua Health Limited has licensed a vaccine (Renogen) for use in Canada and the United States in Atlantic salmon. This vaccine is a heterologous vaccine, consisting of a preparation of the bacterium *Arthrobacter sp. nov.* It appears 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 has undergone 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 IDFG and 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 on these stocks being reared at NMFS facilities at Manchester, strongly suggest that a one-time administration of this vaccine fails to protect these fish from BKD, particularly in the second and third years of culture.

The objectives of the studies carried out under the 2000-2001 and 2001-2002 work plans were to measure the efficacy of Renogen to protect chinook salmon from subsequent challenge with a virulent strain of *R. salmoninarum*, and subsequently to test

the efficacy of other whole-cell vaccine combinations. These included a whole cell (bacterin) vaccine consisting of an attenuated (less virulent) strain of *R. salmoninarum*, MT239, and a combination of Renogen and MT239. This work was carried out in conjunction with other studies in our laboratory where we are seeking to understand and characterize important virulence factors expressed by *R. salmoninarum* and how they might be exploited to form the basis of a more effective BKD vaccine (“Role of Major Soluble Antigen in Pathogenicity of Bacterial Kidney Disease,” USDA NRICGP project # 00-35204-9225).

Materials and Methods

Experiments carried out under FY 2000-2001 work plan

Renogen, trials 1 and 2 --“Renogen” is a commercially available BKD vaccine, produced by Aqua Health Ltd. that consists of a live preparation of the organism *Arthrobacter sp. nov.* Vaccination of Atlantic salmon with this preparation is reported to offer considerable protection against subsequent BKD challenge. While the live bacterium persists up to 30 days after inoculation, it is not clear from available data as to the type of immunity induced against *R. salmoninarum*. In early 2000, the IDFG Eagle Fish Health Laboratory carried out initial safety studies in chinook salmon and found that no adverse effects resulted from the vaccine (K. Johnson, IDFG. pers. commun.). At the same time we initiated preliminary efficacy studies with the vaccine to determine its usefulness in decreasing the incidence of BKD in captive broodstock.

Approximately 1,800 George Adams fall chinook juvenile salmon were IP vaccinated against *Vibrio* (Aqua Health Vibrogen-2 *Vibrio anguillarum-ordalii* bivalent bacterin), and held 3 weeks prior to smolting. The fish were then transferred to the MRS, and allowed to acclimate for at least 30 days. Fish were fed a standard non-medicated diet. During the acclimation period, a random sample of fish was collected and their kidneys were screened for the presence of *R. salmoninarum* infection by ELISA and by reverse transcription-polymerase chain reaction (RT-PCR) (Rhodes et al. 1998). Following acclimation, fish were randomly divided into two groups. Nine hundred fish were anaesthetized (50 mg/L MS 222) and IP-vaccinated with 0.1 ml of Renogen, reconstituted and diluted according to the manufacturer’s specifications. The remaining 900 fish were mock-vaccinated by similar IP injection with 0.1 ml PBS. The groups were kept separate and observed for handling mortality. After 30 days, 300 fish from each of the Renogen-vaccinated and mock-vaccinated groups were inoculated IP with a dose of *R. salmoninarum* (ATCC strain 33209) designed to produce clinical, acute BKD (approximately 1×10^6 bacteria/10-12 g fish) within 60 days. These fish were then distributed into six 5-ft circular tanks (100 fish/tank). The remaining 300 fish from both groups were held as mock challenged controls.

In a separate experiment, approximately 90 days following the initial Renogen vaccination, the challenge experiment was repeated on remaining fish that were initially *Vibrio*-vaccinated followed by Renogen or mock-vaccination. There was no Renogen

booster administered. This experiment was carried out to determine if long-lasting protection was afforded by the Renogen vaccine.

Following challenge in both sets of experiments, fish survival was monitored, and all mortalities were necropsied. To verify death by BKD, kidney tissues were examined by fluorescent antibody test (FAT) to determine levels of *R. salmoninarum*, and ELISA assays were performed on fish with inconclusive FAT values. Representative fish from all treatments were also routinely sampled and analyzed (FAT and ELISA) for the presence of the bacterium, in order to monitor changes in bacterial load after treatment.

MT239 (attenuated *R. salmoninarum*), trials 1 and 2--These trials utilized the same stock of *Vibrio*-vaccinated fish as for the Renogen 1 and 2 trials, and tested the ability of a bacterin produced from an attenuated strain of *R. salmoninarum* to vaccinate fish. This study has been combined with other work in progress in our laboratory concerning the role of the major soluble antigen (MSA or p57) expressed by *R. salmoninarum* in the bacterium's pathogenesis (O'Farrell and Strom 1999; Senson and Stevenson 1999; Rhodes et al., submitted). As part of a continuation of this study the effect of MSA on immune function is being characterized. This includes a comprehensive testing approach to compare the relative efficacies of MSA-defective (strain MT239) and wild type cell lines in eliciting an immune response to bacterial challenge.

A prime-boost strategy was used for these studies, using live and killed (bacterin) vaccine preparations. Bacterin inocula were prepared by killing cultures of *R. salmoninarum* with 3% (v/v) formalin overnight at 15°C, followed by washing with PBS. For the first trial, 100 fish were initially vaccinated with 0.1 ml PBS (mock vaccination), 0.1 ml killed MT239 ($\sim 3 \times 10^3$ cells/fish), or live MT239 ($\sim 2 \times 10^4$ cells/fish), delivered by IP injection. After 6 weeks, the fish were given a booster vaccine consisting of the same preparations at higher doses (killed MT239: 1×10^4 cells/fish; live MT239: 8×10^4 cells/fish). Four weeks after the boost, 50 fish from each group were challenged with a virulent *R. salmoninarum* strain ($\sim 5 \times 10^4$ cells/fish, in 0.1 ml, delivered IP), while the remaining 50 fish were injected with 0.1 ml peptone-saline diluent. The fish were monitored daily and necropsies performed on all mortalities. All kidneys were analyzed by BKD-ELISA.

In the second MT239 trial, excess fish originally vaccinated with *Vibrio* and Renogen 8 months prior were used. In this trial, 100 fish each were immunized with 0.1 ml PBS, killed MT239 (1×10^6 cells/fish), or live MT239 (1×10^5 cells/fish). After 4 weeks, the fish were boosted with the same dosages and formulations. After an additional 4 weeks, half of the fish in each group were challenged with a virulent strain of *R. salmoninarum* (1×10^6 cells/fish), while the other half were injected with an equal volume of peptone-saline. Mortalities were processed as described previously.

Experiments carried out under FY 2001-2002 work plan

Under the FY 2001-2002 work plan, additional trials were performed to determine the efficacy of Renogen, alone or in combination with the MT239 bacterin. For these experiments, approximately 1500 George Adams fall chinook juvenile salmon were IP-vaccinated against *Vibrio* (Aqua Health Vibrogen-2 *Vibrio anguillarum-ordalii* bivalent bacterin) prior to smolting. In order to minimize co-variance between tank and vaccine effects, these fish were PIT tagged (Prentice and Flagg 1987), allowing co-habitation of the various vaccine groups receiving the same challenge. Approximately one month after the *Vibrio* vaccination and PIT tagging, the fish were transferred to the Manchester Research Station, and allowed to acclimate in seawater for 8 weeks. Fish were fed a standard non-medicated diet. During the acclimation period, fish were randomly sampled and kidney tissues analyzed by ELISA for the presence of *R. salmoninarum*.

Renogen vaccination followed by a waterborne challenge—One hundred fish were vaccinated with Renogen as described previously, while another 100 were mock-vaccinated. Twenty eight days after vaccination, these fish were divided into two groups and half were challenged using a waterborne (immersion) delivery of the pathogen to mimic a more natural route of horizontal infection. The challenge consisted of immersing the fish in a static bath of virulent *R. salmoninarum* at a concentration of 1×10^8 bacteria/ml for 2 hours. Previous studies in our laboratory have shown that this level of exposure is sufficient to infect 1 g chinook salmon, with overt disease becoming apparent within 45-60 days. Control (mock-challenged) fish were treated in an identical manner with the exception that they were exposed only to the carrier medium (KDM2 broth) used to make up the bacterial challenge. After the challenge, the water flow was resumed to the 5-foot tanks (50 fish/tank), and survival was monitored.

Renogen and MT239 combination vaccination--In these trials, we further tested Renogen alone and in combinations with MT239 in order to determine if a combinatorial strategy would increase the protective immune response against subsequent *R. salmoninarum* challenge. As before, a prime-boost strategy was used.

After seawater acclimation, 300 fish were divided into three groups. A total of 100 fish were IP vaccinated with Renogen (0.1 ml, 1×10^5 CFU/fish in PBS) following anaesthetization with 50 mg/L MS222. A second group of 100 were vaccinated with a combination of Renogen (1×10^5 CFU/fish) and killed MT239 (5×10^6 cells/fish). A third group of 100 were mock-vaccinated by similar IP injection with PBS (0.1 ml). The groups were kept separate and observed for mortality. After 4 weeks, the fish were boosted with an inoculum identical to the prime vaccination. Two weeks after the booster vaccination, half of the fish from each group were challenged with virulent *R. salmoninarum* via IP injection (5×10^4 cells/fish), while the other group was mock-challenged with peptone-saline. Challenged fish from each vaccination group were equally distributed to four 5-ft circular tanks, with fish from all 3 vaccination groups commingled in each tank to eliminate covariance between vaccination and tank effects. The same procedure was used to mix the unchallenged fish in four separate tanks. Immediately after challenge and before placement into the tanks, the PIT tags were read

and recorded. Mortalities were removed daily during the trial, the PIT tags read to determine which group the fish belonged to, and basic necropsies and bacteriology performed as described above. In addition, all kidney tissues were collected and analyzed by BKD-ELISA.

Results

FY 2000-2001 work plan

Renogen, trials 1 and 2--In the first trial with Renogen, mortality associated with the challenge dose of *R. salmoninarum* began ~19-20 days after the challenge injection. The mortality curve of the Renogen vaccinated fish was slightly but significantly delayed as compared with the non-vaccinated control group, by approximately 5 days (Figure 1). However, there was little if any difference in the slope of the mortality curves and all fish in both challenged groups eventually died by 6 weeks post-challenge. In the second trial, there was no difference in either the onset or rate of mortality between the Renogen-vaccinated and mock-vaccinated groups following acute challenge with *R. salmoninarum* (data not shown).

MT239 (attenuated *R. salmoninarum*), trials 1 and 2--The utility of live or killed MT239 as a prophylactic against acute intraperitoneal challenge by *R. salmoninarum* was tested in these trials. As with Renogen, vaccination with live MT239 provided limited but significant protection (Figure 2A and 2B). No significant difference in protection between live and killed MT239 was observed (Figure 2B).

In these trials, the presence or absence of *R. salmoninarum* was confirmed by performing BKD-ELISA on kidney tissue taken from fish at necropsy, including mock challenged fish and fish sampled before initiation of the experiment (time 0). The high ELISA values measured in the challenged fish kidney tissue strongly supports the conclusion that mortality was due to infection by *R. salmoninarum*. In contrast, the low ELISA values for kidneys from the starting population as well as the survivors among the mock-challenged fish indicated that these fish were uninfected or carried negligible burdens of *R. salmoninarum* (Figure 3A and 3B).

FY 2001-2002 work plan

Renogen vaccination followed by a waterborne challenge--To date, most of our attempts to experimentally infect salmon with *R. salmoninarum* have been by direct intraperitoneal injection of a known quantity of the bacterium. The advantages include the ability to give each fish the same dosage of the pathogen as well as to be able to predict how long it will take for disease to cause mortality based on prior experience. However, this is obviously an “unnatural” route and raises the concern that such an acute infection would overwhelm the protective effects of any vaccine. Therefore, an attempt was made to measure the efficacy of Renogen to prevent *R. salmoninarum* infections when the bacterium was introduced to fish via a waterborne challenge, which would more closely resemble horizontal exposure of fish to the pathogen.

In this trial, one group of juvenile chinook salmon were vaccinated with Renogen (no booster vaccination) while a second group was mock-vaccinated. After 28 days, the fish were challenged by immersion in a bath of viable, virulent *R. salmoninarum* cells as outlined in the methods. Previous experience had demonstrated that this method of infection was sufficient to initiate BKD successfully in very young chinook (~ 1 g body weight) 45-60 days post-challenge (unpublished information in our laboratory). Results of this experiment demonstrated no difference in time of onset or cumulative mortality between vaccinated and mock-vaccinated fish. However, there was also no difference in mortality between fish challenged via experimental exposure to *R. salmoninarum* and those that were not. Retrospective analysis demonstrated that individuals within this particular group of chinook already were infected with *R. salmoninarum* prior to the experiments. This was likely due to vertical infections, although overt BKD did not manifest itself until the fish were in the middle of the experiment. Therefore, if a single Renogen vaccination was to have any therapeutic value, it might be expected that the vaccinated, mock-challenged group would have had a higher survival rate than the mock-vaccinated, mock-challenged group, which was not the case.

Renogen and MT239 combination vaccination--In the trials carried out under the 2000-2001 work plan, we demonstrated that individually Renogen and MT239 provide limited prophylactic protection against acute *R. salmoninarum* challenge. Therefore, we hypothesized that a combination vaccine containing both Renogen and MT239 might afford greater protection. Furthermore, the therapeutic effects of Renogen or MT239 had not yet been investigated. The recognition that the fish used in this year's trials were naturally infected with *R. salmoninarum* through vertical transmission allowed us to examine these possibilities. In this trial, yearling chinook salmon were immunized and boosted with Renogen or Renogen with MT239 as described in the Methods. Subsequently, these fish were mock-challenged or challenged with virulent *R. salmoninarum* by acute intraperitoneal inoculation. Because the fish had been naturally infected with *R. salmoninarum* prior to the start of the experiment (kidney ELISA at the start of the experiment: mean = 0.715, range = 0.196 to 3.239, n = 21), mortalities were observed among mock challenged fish throughout the experimental period (Figure 4). Although mortalities for both whole cell vaccinated groups declined after day 98, only the survival curve for the combination vaccinated fish was significantly different from the control curve. Among fish challenged with *R. salmoninarum*, those vaccinated with both whole cell vaccines were significantly protected, but the degree of protection with the Renogen or the combination vaccine was statistically identical (Figure 5).

High kidney ELISA values confirmed that both mock challenged and challenged mortalities were the result of *R. salmoninarum* infection (Figure 6). When the kidney ELISA values of mock challenged fish surviving the experiment were determined, there was a striking difference among the vaccination groups. The fish treated with the combination vaccine exhibited significantly lower kidney ELISA values than the PBS or Renogen vaccinated fish (Figure 7). This suggests that the combination vaccine had an effect on the preexisting *R. salmoninarum* infection. This analysis is strengthened when the relative percent survival (RPS; Amend 1981) of the different groups was calculated and compared after no mortalities occurred in the corresponding control group for at least

3 consecutive days or at the end of the experiment (Figure 8). The RPS values of the challenged fish vaccinated with the combination vaccine were measurably higher than either the MT239 or Renogen-vaccinated groups. This difference was even more striking with the RPS values obtained with the naturally infected groups. Here the combination vaccine group had an RPS value of ~34% compared with ~23% for the Renogen-vaccinated group. These results also show that the combination vaccine may be able to reduce disease severity in already infected fish.

Discussion

The vaccine trials described here were designed to determine the efficacy of a commercial BKD vaccine, Renogen, to protect juvenile chinook salmon from BKD. Renogen consists of a heterologous bacterium (*Arthrobacter*), and its effectiveness lies in the fact that some form of cross-reacting immune response is mounted in Atlantic salmon against it. This was extended to determine whether an attenuated strain of *R. salmoninarum* could also serve as a whole cell bacterin. MT239 is defective in expression of MSA (major soluble antigen; also called p57), a protein that is involved in virulence of *R. salmoninarum* in an as yet undefined way (O'Farrell and Strom 1998). Our trials with MT239 in chinook salmon were stimulated by findings from other researchers who demonstrated that vaccination of Atlantic salmon with bacterins prepared from wild-type strains, where MSA had been removed, or MT239 resulted in limited protection from BKD (Piganelli et al. 1999).

Our studies demonstrated that injection vaccination with either Renogen or MT239 provides a limited protection against acute intraperitoneal (IP) challenge with *R. salmoninarum* in uninfected yearling chinook salmon. Recognizing that IP challenge is not the natural route of infection and may in fact overwhelm the juvenile immune system and/or any vaccine-induced immunity with an extreme bacterial load, we attempted one trial where an immersion challenge was used. Unfortunately, this trial did not demonstrate any difference in mortality in challenged vs. mock-challenged control fish, and therefore the results are inconclusive. However, it should be pointed out that this group of fish were already infected naturally with *R. salmoninarum* and did exhibit some BKD-related mortality in all groups. Therefore, one can conclude that Renogen alone in these fish did not result in any therapeutic effect in control of existing BKD.

In our trials using a combination of Renogen and MT239 vaccination of yearling chinook, vaccinated fish given an acute *R. salmoninarum* challenge had a significantly longer survival time than the mock vaccinated group. This survival time was marginally higher than was seen in acutely challenged fish vaccinated with either Renogen or MT239 alone. However, the more interesting result is the decrease in mortality and kidney ELISA values that was measured in fish already naturally infected with *R. salmoninarum*. which suggests that a combination vaccine of Renogen and MT239 may be useful as both a prophylactic and therapeutic agent against BKD. This result is encouraging from a vaccine development standpoint in that it demonstrates that survival of vertically-infected salmon may be increased through a vaccination protocol. We are continuing to understand the cellular mechanisms behind this increase in survival, and to

incorporate a program of vaccination combined with other chemotherapeutic treatment regimens, such as the macrolide antibiotic azithromycin. The goal of a multi-pronged approach is to clear the pathogen from vertically-infected juvenile captive broodstock, thereby breaking the cycle of *R. salmoninarum* infection and chronic BKD in these stocks, and increasing the overall in-culture survival.

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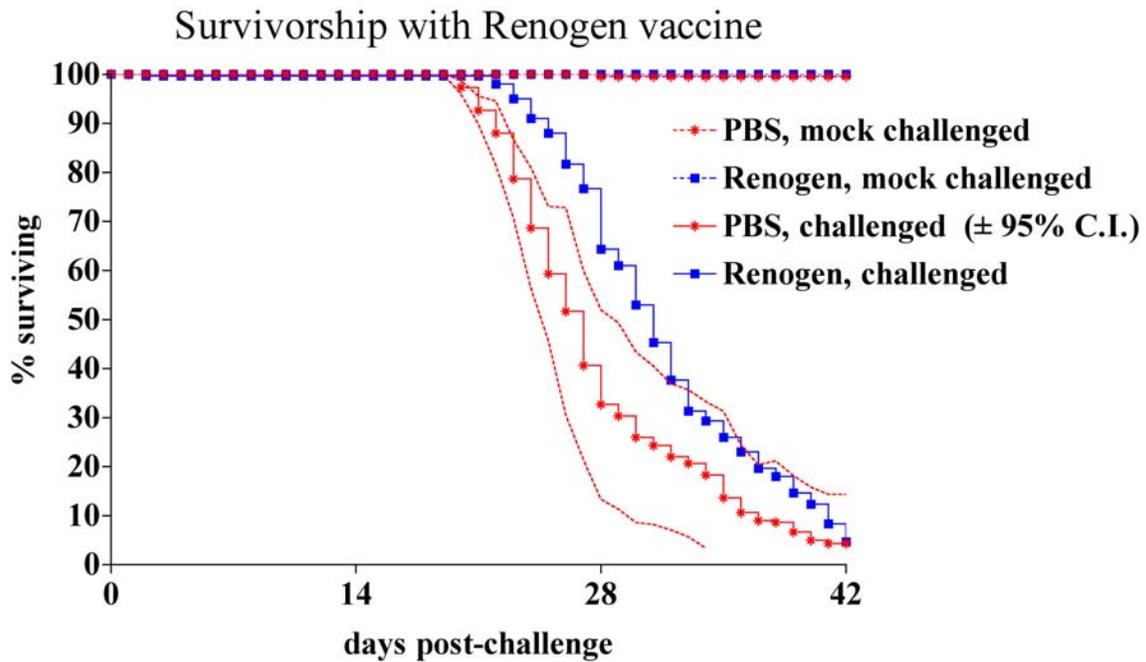


Figure 1. The percentage of yearling chinook surviving intraperitoneal challenge with virulent *R. salmoninarum* over time after vaccination with Renogen or PBS (mock-vaccinated) is shown. The smooth lines represent the $\pm 95\%$ confidence interval for the mock-vaccinated and challenged group.

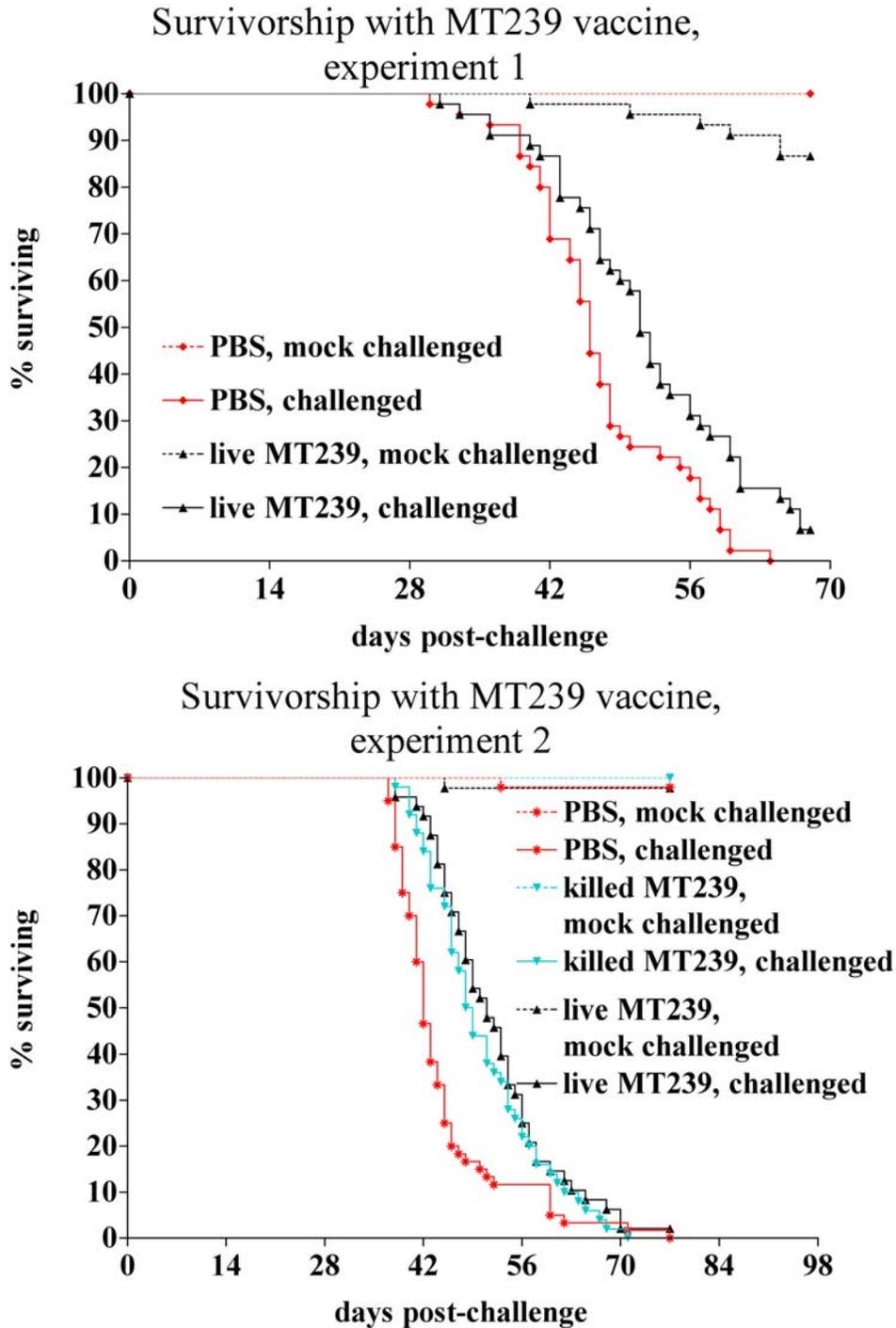
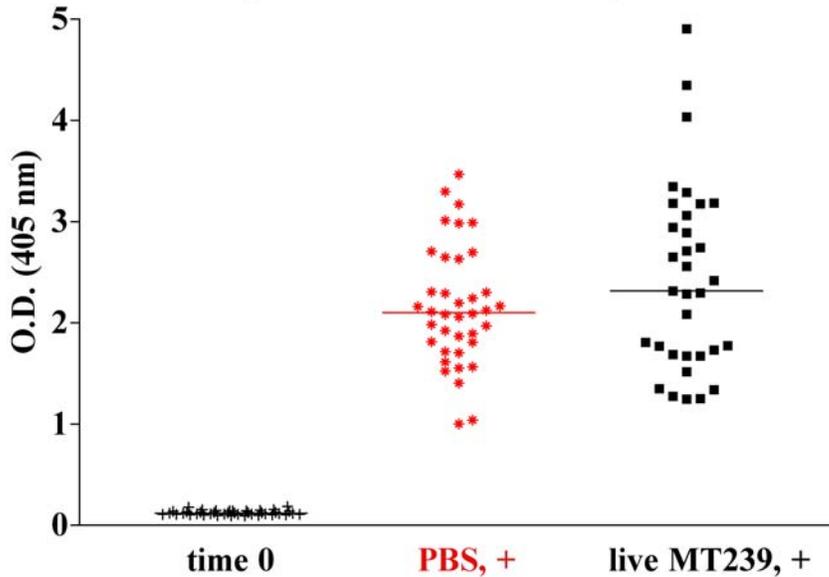


Figure 2. The percentage of yearling chinook surviving intraperitoneal challenge with virulent *R. salmoninarum* over time after vaccination with live or killed *R. salmoninarum* strain MT239 and PBS (mock-vaccinated) is shown for Trial 1 (top panel) where only live MT239 was used as a vaccine candidate and Trial 2 (bottom panel) where the efficacy of live and dead (formalin-fixed) MT239 were compared as vaccine candidates.

Kidney ELISA values for time 0 sacrifices
and challenged mortalities (+), experiment 1



Kidney ELISA values of mock challenged survivors (-)
and challenged mortalities (+), experiment 2

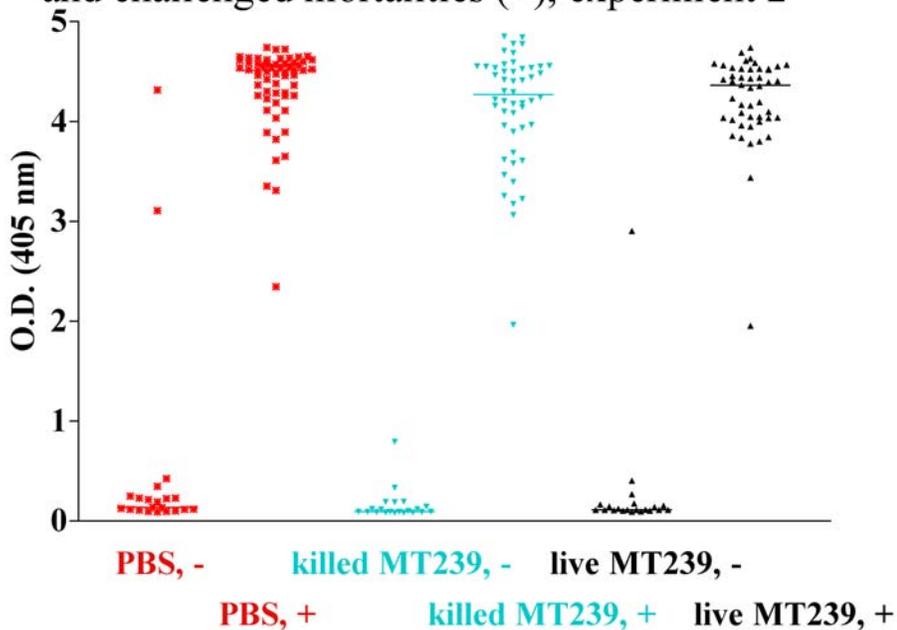


Figure 3. Kidney ELISA values obtained from time 0 sacrifices and mortalities after challenge in the MT239 vaccine trials (see figure 2) for Trial 1 (top panel) where vaccination with live MT239 was tested and Trial 2 (bottom panel) where vaccination with live and dead MT239 was compared.

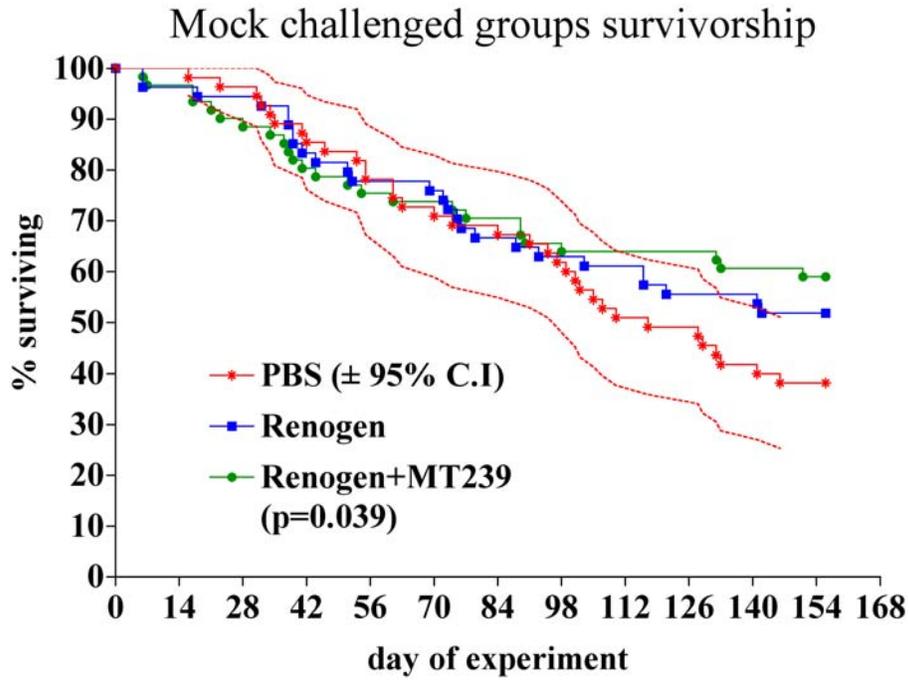


Figure 4. The percentage of surviving yearling chinook after vaccination with MT239 or a combination of MT239 and Renogen. These fish were naturally infected with *R. salmoninarum* prior to vaccination.

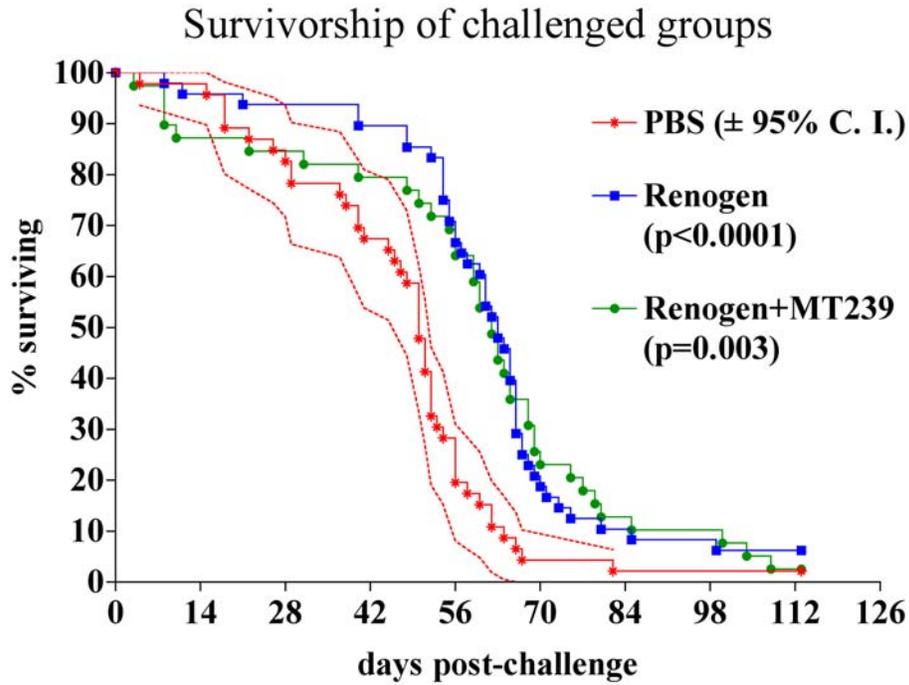


Figure 5. The percentage of surviving yearling chinook after vaccination with MT239 or a combination of MT239 and Renogen followed by an acute challenge with virulent *R. salmoninarum*. These fish were naturally infected with *R. salmoninarum* prior to vaccination.

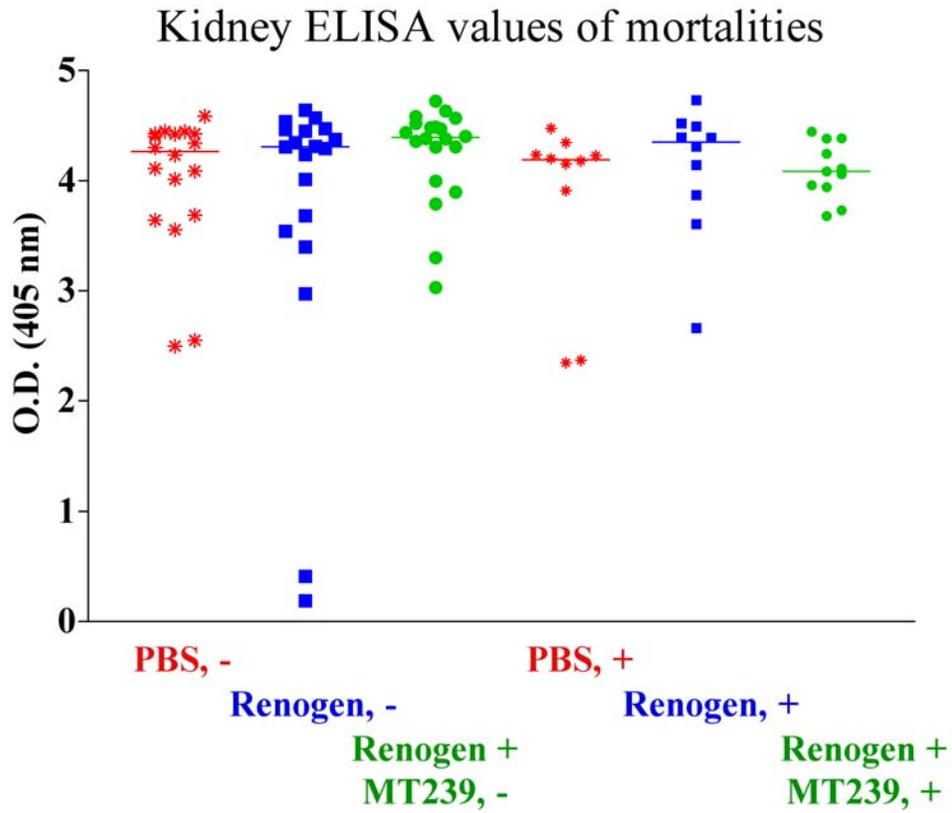


Figure 6. Kidney ELISA values obtained from mortalities in both challenged and mock-challenged chinook after various vaccination protocols as described in the Results section “Renogen and MT 239 combination vaccination”.

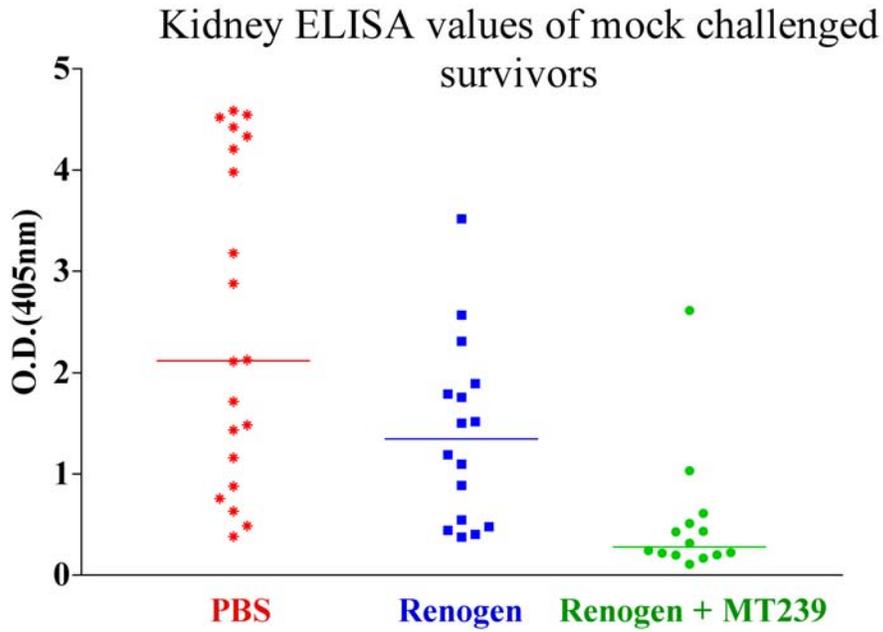


Figure 7. Kidney ELISA values obtained from mock-challenged surviving chinook after various vaccine protocols as described in the Results section “Renogen and MT 239 combination vaccination”.

Comparison of RPS Values for Whole Cell Vaccines

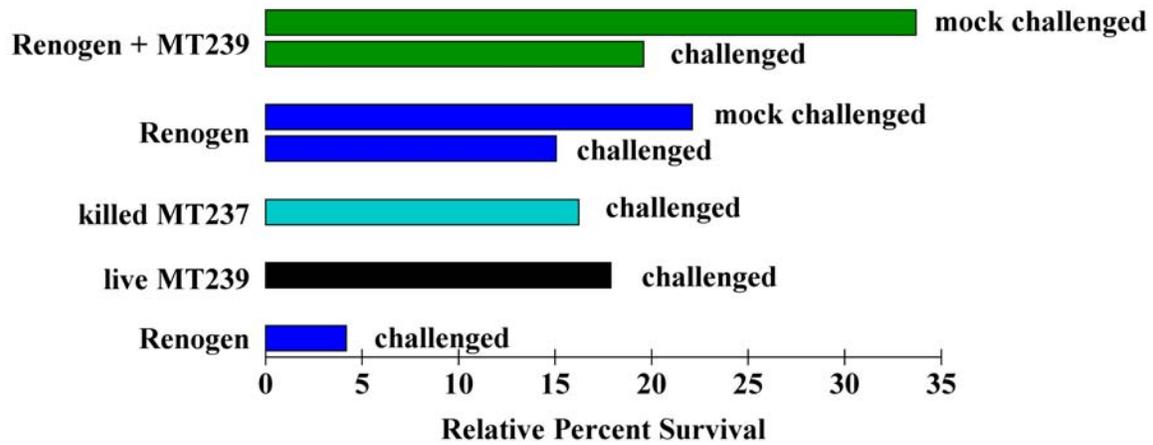


Figure 8. Relative percent survival (RPS) values compared between chinook vaccinated with MT239, Renogen, or both, and then either challenged with virulent *R. salmoninarum* or mock-challenged. The data in this figure includes most of the trials described in the previous figures (see Results).

**TASK 10. USE OF ANTIBIOTICS AND NEW VACCINES TO REDUCE
MORTALITY FROM BACTERIAL KIDNEY DISEASE IN CHINOOK SALMON**

(PROGRESS REPORT: 1 JUNE 2001 THROUGH 31 MAY 2002)

**(Experiment B. Evaluation of Toxic Effects of Long-term Prophylactic Use of
Azithromycin and Erythromycin)**

by

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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).

There is no vaccine available to protect salmon from infections with *Renibacterium salmoninarum*, the causative bacterium of BKD. Erythromycin has been the primary antibiotic used by fish culturists in an attempt to prevent and control *R. salmoninarum* (Elliott et al. 1989), administered orally through feed or by injection of maturing adults. However, while use of erythromycin usually results in short-term health improvement of infected fish, it fails to eliminate the infection and symptoms of disease completely, which often return after ending treatment. In addition to erythromycin another macrolide antibiotic, azithromycin, has been tested for the treatment of BKD in captive broodstock salmon. In experiments carried out between 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 (Bandin et al. 1993, Gutenberger et al. 1997).

Long-term prophylactic use of antibiotics should be avoided where possible. In order to reduce antibiotic treatment of BKD, effective vaccines or other non-antibiotic-based therapeutics will have to be developed and used. 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, or bacterins, heat-treated whole fixed cells (to reduce the amount of p57 protein on the surface, a putative major virulence factor), and purified p57.

Recently, Aqua Health Limited has licensed a vaccine (Renogen) for use in Canada in Atlantic salmon. This vaccine is a so-called heterologous vaccine which consists of a preparation of the bacterium *Arthrobacter sp. nov.* It appears to induce a cross-reacting immune response which offers protection against *R. salmoninarum* infection. The bacterium is administered as a live culture, and delivered in an intraperitoneal injection. The vaccine has undergone safety testing in chinook salmon presmolts at the IDFG Eagle Fish Health Laboratory in early 2000. Under this proposal, the vaccine will be administered to chinook salmon presmolts. After smolting, the vaccinated fish will be experimentally-challenged with a virulent strain of *R. salmoninarum* to determine the efficacy of the vaccine to protect chinook salmon from BKD. Besides Renogen, preliminary vaccine trials using attenuated strains of *R.*

salmoninarum currently under study by the Northwest Fisheries Science Center will also be initiated under this proposal.

The long-term prophylactic administration of erythromycin is commonly used to reduce losses due to BKD. 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. Until an effective vaccine becomes available, erythromycin and/or azithromycin will continue to be used. The approach is to design treatment regimens which avoid these toxic reactions and reductions in reproductive success having first determined the causes of the reactions and conditions under which they appear. A long-term study on potential toxic reactions after prophylactic use of erythromycin and azithromycin will be completed, together with the verification that limited prophylactic use of the more experimental azithromycin in captive broodstock rearing programs does not result in its release in effluent. In addition, vaccine trials will be initiated to determine the efficacy of a new commercial BKD vaccine as well as experimental vaccine preparations to induce a protective immune response.

Work completed

The three main objectives of this experiment are: (i) determine how captive fall chinook salmon broodstock gonad development, gamete viability, and survival of the progeny through the swim-up stage are affected by long-term prophylactic administration of erythromycin or azithromycin; (ii) measure, if the experimental fish are found to be naturally infected with *R. salmoninarum*, the effects of two erythromycin treatment regimens (two or four treatments annually) on BKD incidence from the first feeding fry through mature adult life history stages; and (iii) determine whether erythromycin toxicity responses are related to treatment frequency. This aspect of the study will include a measurement of residual tissue concentrations and will document underlying tissue damage associated with the syndrome. While the study focuses on erythromycin as it is the current antibiotic of choice and the one apparently causing the severe toxic side effects, the long-term effects of azithromycin treatment will also be concurrently assessed.

Phase I: First feeding to smolt

During January 1999, approximately 3,150 George Adams fall chinook salmon were transferred to the Big Beef Creek Hatchery. Prior to initiation of exogenous feeding, 400 fish were randomly stocked into each of 14 isolation tanks (two tanks per experimental treatment). Using this experimental design, the following feedings were carried out:

- No treatment.
- Erythromycin administered orally at a rate of 100 mg/kg fish body weight per day for 28 days, or azithromycin administered orally at a rate of 30 mg/kg fish body weight per day for 14 days. The first treatment was administered at initiation of exogenous feeding, the second treatment administered during sexual differentiation (2 g average weight), and the third just prior to smoltification (ca. 7 g average weight).
- Erythromycin administered orally at a rate of 100 mg/kg fish body weight per day for 28 days, or azithromycin administered orally at a rate of 30 mg/kg fish body weight per day for 14 days, with the first treatment administered during sexual differentiation (2 g average weight), and the second just prior to smoltification (ca. 7 g average weight).
- Erythromycin administered orally at a rate of 100 mg/kg fish body weight per day for 28 days, or azithromycin administered orally at a rate of 30 mg/kg fish body weight per day for 14 days, with the only treatment administered just prior to smoltification (ca. 7 g average weight).

At the beginning of the trial and following each treatment, fish from each experimental tank were collected for measurement of tissue antibiotic concentrations and for histological evaluation of brain, heart, liver, spleen, stomach, pyloric caecae, and intestine for abnormalities associated with erythromycin or azithromycin toxicities. The collected specimens are being analyzed following methods described in the work plan.

Phase 2: Smolt to maturity

Phase 2 of the study was initiated in June 1999. One hundred twenty fish from each tank were PIT tagged and divided randomly into three groups of 40 fish each, which were combined as follows:

- Tank 1: Forty fish from pre-smolt treatments 1-7, replicate A
- Tank 2: Forty fish from pre-smolt treatments 1-7, replicate A
- Tank 3: Forty fish from pre-smolt treatments 1-7, replicate A
- Tank 4: Forty fish from pre-smolt treatments 1-7, replicate B
- Tank 5: Forty fish from pre-smolt treatments 1-7, replicate B
- Tank 6: Forty fish from pre-smolt treatments 1-7, replicate B

Two tanks of fish were then assigned to each of the experimental treatments. Using this experimental setup, the following feedings were carried out:

- No treatment
- Erythromycin administered orally at a rate of 100 mg/kg/d for 28 d, twice per year until they matured (November-December 2001).
- Erythromycin administered orally at a rate of 100 mg/kg/d for 28 d, four times per year until matured (November-December 2001).

During August 2000, fish from each experimental tank were collected for measurement of tissue antibiotic concentrations, and for histological evaluation of brain, heart, liver, spleen, stomach, pyloric caecae, and intestine for abnormalities associated with erythromycin or azithromycin toxicities. The collected specimens were preserved for analysis at the end of the study, pending review of the experimental results.

Most of the fish matured during fall 2001. Females from each treatment group were paired with males in a factorial arrangement which will permit detailed evaluation of gamete quality. Fertilized eggs from each mating were incubated in isolation to hatching. Sub-samples of eggs have been examined to measure fertilization and survival to the eyed stage. Fry were counted, measured, and weighed at swim-up, and samples taken for erythromycin analysis.

Work to be completed

Phase 1: First feeding to smolt stage

All samples collected during this phase have been analyzed to determine the concentration of erythromycin or azithromycin in the tissues. Preliminary histological evaluation of fish subjected to the experimental treatments has been completed. Additional samples are now being independently evaluated to confirm the preliminary results. A final report will be provided in the FY 03 Annual Report.

Phase 2: Smolt to maturity

Samples collected during spawning to evaluate the effects of the various treatment regimens (i.e., pre- and post-smolt treatments) on BKD prevalence, drug clearance rates, and organ histology are currently being analyzed in the laboratory. Laboratory work will be completed by July 2002. A final report will be included in the FY 03 Annual Report.

TASK 11. INBREEDING.

(PROGRESS REPORT: 1 JUNE, 2001 THROUGH 31 MAY, 2002)

by

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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. 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. in review).

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).

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 reduce risk of inbreeding depression and domestication simultaneously.

Approach

In this ongoing research project, three basic hypotheses are being tested:

- H_{01} : 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.
- H_{02} : 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).
- H_{03} : 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 2001-2002, 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 Fishery and Aquatic Sciences Hatchery (UWH), the site of releases of inbred progeny. We currently have no captive experimental fish in culture at the Manchester Research Station (MRS), the site of captive rearing in marine net-pens. Essentially, one complete generation of experimental inbreeding will be complete with the maturation of adult inbred progeny (through age 4) in November 2003. This report summarizes current progress and some new preliminary analyses for the study.

Work Completed

To date we have in this study:

- 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, an experimental population at Grovers Creek Hatchery structured of 96 full-sib families nested within 30 half-sib families;
- released 257,093 of these fish from Grovers Creek Hatchery to sea in 1995, 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 6 experimental groups;
- established at MRS in marine net-pens 3618 1997-brood PIT-tagged smolts, composing a total of 28 families in 6 experimental groups;
- released to sea from UWH 85,111 1998-brood CWT smolts, composing a total of 70 families in 4 experimental groups;
- established at MRS in marine net-pens 2088 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 120 adults returning to UWH in 1999, 2000, and 2001 (total return of 5000). Most tags have been decoded. We constructed no matings in these years from these fish because too few experimental females were available for a full mating design (most second-generation matings for inbreeding will be made in 2002 with the return of four-year old fish from the 1998-brood fish released from UWH in 1999).

We summarized preliminary 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. These data are undergoing further analyses, which we will summarize in a final report by June 2004.

Analysis of experimentally inbred progeny

Here we summarize some analyses conducted 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.

Early survival--We measured survival rates at three stages during early development in 1997- and 1998-brood study progeny at UWH. The mean survival rate in the 1997 brood was lower in both half-sib and full-sib mating groups at all stages than in the unrelated mating group (Table 1). Compared with the unrelated mating group, mean survival in the half-sib mating group at the eyed, hatch and alevin stages decreased, but mean survival rates in half-sib and full-sib mating groups were similar at all stages. A decrease in the mean survival rate was also observed in both half-sib and full-sib mating groups compared with the unrelated mating group at all stages in the 1998 brood (Table 1). Compared with the unrelated mating group, mean survival in the half-sib mating group at the eyed, hatch and alevin stages decreased; however, mean survival rate in the full-sib mating group was higher than in the half-sib mating group at all stages. Nested ANOVA for transformed survival rate revealed that survival rates at each stage were similar for the

two broods, and that none of changes across groups in the survival rate was significant (Table 2).

Decreased mean survival rate relative to the unrelated mating group was observed at all developmental stages in both half-sib and full-sib groups in the two broods, although the effects of inbreeding on survival rate were significant only in the 1998-brood half-sib mating group. Decreases detected in this study were not significant, in disagreement with the results for rainbow trout by Gjerde et al. (1983), but in agreement with the results for rainbow trout by Kincaid (1976a, b), who found no significant effect of inbreeding on egg hatchability at $\Delta F = 0.25$ and $\Delta F = 0.375$.

With the exception of 1998-brood full-sib mating group, which showed similar decreases at all stages in survival, a decrease in survival in the inbred groups was more pronounced after the eyed stage. More pronounced decreases in survival at later developmental stages were also observed in rainbow trout by Kincaid (1976a, b), who found nonsignificant reductions in embryos and egg hatchabilities but a significant reduction in percent fry survival under inbreeding. Similarly, Su et al. (1996) also noted a tendency for inbreeding depression for body weight to increase with advancing age, suggesting that close inbreeding might have more severe effects during stressful developmental transitions in salmonids, such as during the hatching process or at first feeding.

Significant effects of inbreeding on survival rate were found for the half-sib mating group in the 1998 brood by a permutation test. However, the nested ANOVA failed to reveal significant effects of inbreeding among all groups. Low power due to insufficient number of families within each group may account for the failure to detect significant differences between different mating groups in this study. In fact, retrospective power analyses showed that the greatest power among these three separate nested ANOVAs (Table 2) is only approximately 10%, suggesting that a larger number of families within each group would be required to detect a significant effect of inbreeding under this pattern of variation.

There was no apparent linear relation between inbreeding depression in survival rate and the level of inbreeding. These results are similar to those obtained from rainbow trout by Kincaid (1976a) and Gjerde et al. (1983). Similarly, there also seemed to be no apparent linear relation between variance of the survival rate and the level of inbreeding at any developmental stage. Several factors could interfere with the effects of inbreeding and obscure a linear relationship between the effects of inbreeding and the levels of inbreeding. For instance, diverse parental genetic backgrounds may lead to a different response to the same rate of inbreeding in their progeny. The historical tetraploidy in salmonid evolution might also complicate the relationship of survival rate with levels of inbreeding if tetraploidy can “buffer” against inbreeding depression. Finally, the relationship between inbreeding and inbreeding depression may not be linear but exhibit a threshold response if inbreeding accumulates to a level high enough (Frankham 1995).

Marine survival and growth--In 1998 and 1999 we released 1997- and 1998-brood F₂ CWT chinook salmon from UWH. We recovered fish from these releases 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 2090 fish returned to the rack between 21 September and 14 November. Of these fish, 1523 were CWT (235 males and 1,288 females). These fish represented 11 release groups (excluding jacks two years old or younger), summarized in Table 3.

Each of the 1997-brood study releases was very small (these were progeny of 3-year-old females, which occur in low frequency in the Grovers Creek Hatchery population), and these fish were in poor condition at time of release from UWH due to an *Aeromonas* epizootic. As of the end of the 2001, no 1997-brood study fish had been recovered at the UWH rack or in fisheries, according to the PSMFC coded-wire tag database. 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. Returns of full-sibling releases thus far are lower than those of the half-sibling, unrelated, or UW control groups (Figure 1), but returns are not yet complete.

Variation in length and weight for the 1998-brood adult males and females returning to UWH in 2001 is summarized in Table 4. Although sample sizes are small, females from the full-sib mated group tended to have lower weights than those of the other groups. Inbred males were smaller than the UW control group, but the males in the unrelated study group were smaller than any of the other fish in both length and weight.

Developmental asymmetry

One prediction of evolutionary theory is that inbreeding, by reducing overall individual heterozygosity, will tend to promote developmental instability. To evaluate this idea we have sampled experimental fish to detect developmental instability, as measured by bilateral asymmetry. We have completed a preliminary analysis of approximately 750 juvenile 1998-brood fish from the three experimental groups (control, full-sib inbred, half-sib inbred) to detect evidence for effect of inbreeding on developmental asymmetry (Figure 2). The three groups show significant ($P < 0.05$) differences in fluctuating asymmetry (FA, measured here as the absolute differences between left and right side measurements for bilateral characters, corrected for variation among traits in magnitude of deviation = "size corrected") for some but not all traits. The bilateral traits we 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. 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 trait, the groups showed significant ($P < 0.01$) differences in FA; over all traits, the groups also differed significantly (Wilks' $\lambda = 0.91$, $P < 0.0001$).

Surprisingly, however, the pattern of FA was not an expected one: fish from the control (unrelated) matings had the highest degree of FA for all three traits. Fish from

the half-sib and full-sib matings did not differ significantly in FA for pectoral fin ray number or upper gill raker number; fish from the half-sib matings had lower FA than those from full-sib matings in lower gill raker number ($P < 0.05$). At present these results are difficult to explain; future analyses will try to account for additional variation by including spawning date and egg size.

Work to be Completed

Work in 2002-2003 will entail the following elements:

- Collection of experimentally first-generation inbred and control (F_2) 1997- and 1998-brood Grovers Creek stock chinook salmon returning to UWH in September and October 2002. We will continue to collect these fish as they return to UWH until 2003);
- Mating of these fish to perpetuate the experimental lines by creating second-generation inbred (F_3) fish; and
- Culture of the resulting F_3 progeny to the smolt stage in freshwater, followed by release of most fish from UWH, with representatives of each family to be held captive in seawater at the NMFS research stations at Manchester or Mukilteo. Depending upon the number of adults that survive to return to UWH, we will establish up to 50 full-sib families in each of the experimental lines (randomly mated, half-sib mated, and full-sib mated). We will create experimental groups at time of spawning after decoding group-specific coded-wire tags at UWH. We will conduct experimental matings at the end of each spawning day, and culture progeny to the smolt stage at the hatchery. We will mate parents of known lineage from within each line together at random on each spawning date to establish the second-generation experimental groups.

We will collect the following information for four- and five-year old 1997- and 1998-brood adults maturing in autumn 2002 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 their progeny we will collect data through the smolt release in June 2003 on stage-specific survival, growth and development rate, and meristic and morphometric variation. We will mark smolts with group-specific coded-wire tags, with most fish released from the hatchery to sea, and up to 5,000 (with up to 50 representatives from each full-sib family) PIT tagged for grow-out to adulthood in captivity. Collectively, these data will provide a preliminary assessment of second-generation inbreeding effects on early life-history traits and, through examination of the control population, an assessment of relative environmental influences in consecutive generations on these traits.

For next year's report, analysis of inbreeding depression after one generation of inbreeding will be based upon the data collected from 1997- and 1998-brood fish up to 5 years old (4 years old for the 1998-brood fish); this analysis should be complete by June 2003. We expect that evaluations to determine if the three experimental lines differ in

stage-specific survival, growth, development rate, and meristic and morphometric variation will be nearly complete by this time.

For the analyses, we will rely on general linear models and analyses of variance in survival and early growth within and among lines to test the hypotheses stated above and evaluate the effects of inbreeding on these characters, following methods developed by Lynch (1988) and Lynch and Walsh (1998) and described in Hard and Hershberger (1998). If necessary, we will follow these tests with retrospective power analyses identify the magnitudes of observed differences necessary to detect significant inbreeding depression (Cohen 1988). We plan to compare survival and growth among the three experimental groups. For each trait, we will compute the coefficient of inbreeding depression (Lande and Schemske 1985) to provide direct comparison of inbred and control groups. Where it is possible to do so from the survival data, we will estimate the number of lethal equivalents per gamete 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).

We will provide a summary of the comprehensive genetic and phenotypic analyses of the biological data collected from the first-generation parents in our next report. We plan to submit a final report on this project, when one full generation of experimental inbreeding is implemented with the maturation of adult inbred progeny through age 5, by June 2004.

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Table 1. Survival rate at different developmental stages in 1997- and 1998-brood chinook salmon. FS, full-sib mated; HS, half-sib mated; U, unrelated groups. SD, standard deviation. * P < 0.05, F-test for equal variances.

Brood	Developmental stage	Group	Number of families	Survival Mean	rates SD
1997	Eyeing	U	11	82.5	9.8
		HS	11	80.0	14.0
		FS	7	81.4	12.8
	Hatching	U	11	81.1	9.3
		HS	11	75.5	17.0*
		FS	7	74.4	18.2*
	Alevin	U	11	80.6	9.5
		HS	11	75.0	17.1*
		FS	7	73.5	18.7*
1998	Eyeing	U	25	85.0	8.0
		HS	23	76.5	12.4*
		FS	24	80.0	15.3*
	Hatching	U	25	79.3	11.5
		HS	23	66.1	21.0*
		FS	24	74.5	18.9*
	Alevin	U	25	77.3	12.9
		HS	23	64.9	21.6*
		FS	24	72.7	21.5*

Table 2. Nested ANOVA for arcsin-transformed survival rate at different stages in 1997- and 1998-brood chinook salmon, testing for brood and group effects.

Developmental stage	Source of variation	df	MS	F
Eyeing	Brood	1	0.00946	
	Group (Brood)	4	0.13669	0.09
	Error	95	0.10586	1.29
Hatching	Brood	1	0.14336	
	Group (Brood)	4	0.28804	0.86
	Error	95	0.16603	1.73
Alevin	Brood	1	0.22512	
	Group (Brood)	4	0.29809	1.22
	Error	95	0.18474	1.61

Table 3. Releases and preliminary recoveries of experimental fall chinook salmon from the University of Washington School of Aquatic and Fishery Sciences Hatchery for the inbreeding study. FS, full-sib mated; HS, half-sib mated; U, unrelated; UW (UW stock).

Brood year	Treatment	No. released	Release size (g)	Rack recoveries
1996	UW	176,740	14.4	1,439
1997	FS	2,706	8.4	0
1997	FS	150	8.5	0
1997	HS	1,700	7.8	0
1997	HS	2,130	7.4	0
1997	U	3,968	53.8	0
1997	UW	148,527	16.4	389
1998	FS	29,371	----	7
1998	HS	26,021	----	51
1998	U	29,719	----	64
1998	UW control	148,527	----	64

Table 4. Mean fork lengths (FL, mm) and weights (wt., g) of adult 1998-brood males and female chinook salmon recovered at UWH in 2001, summarized by group.

Group	Males		Females	
	FL \pm SE (n)	Wt. \pm SE (n)	FL \pm SE (n)	Wt. \pm SE (n)
Unrelated (U)	644.9 \pm 17.1 (17)	2.834 \pm 0.225 (17)	712.0 \pm 35.9 (4)	4.435 \pm 0.690 (4)
Half-sib mated (HS)	704.1 \pm 20.9 (18)	3.667 \pm 0.311 (18)	691.4 \pm 19.8 (8)	4.045 \pm 0.396 (8)
Full-sib mated (FS)	682.9 \pm 32.1 (8)	3.399 \pm 0.468 (8)	692.3 \pm 7.2 (3)	3.883 \pm 0.188 (3)
UW	736.8 \pm 7.9 (96)	4.506 \pm 0.158 (96)	717.1 \pm 6.3 (84)	4.463 \pm 0.129 (84)

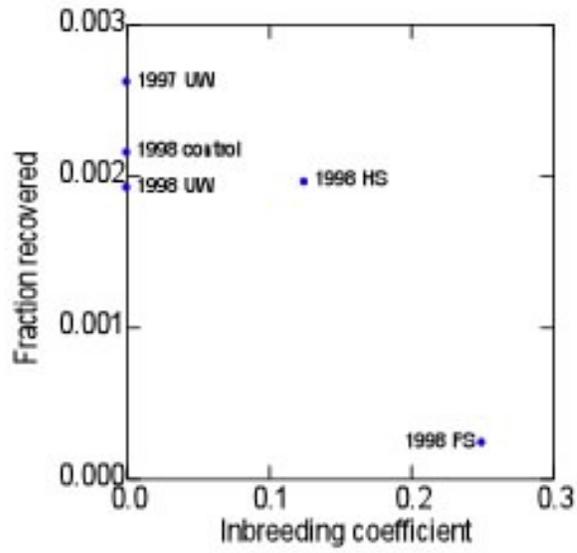


Figure 1. Recoveries of 1997- and 1998-brood chinook salmon at UWH in 2001 as a fraction of numbers released. FS, full-sib mated; HS, half-sib mated. Control = unrelated.

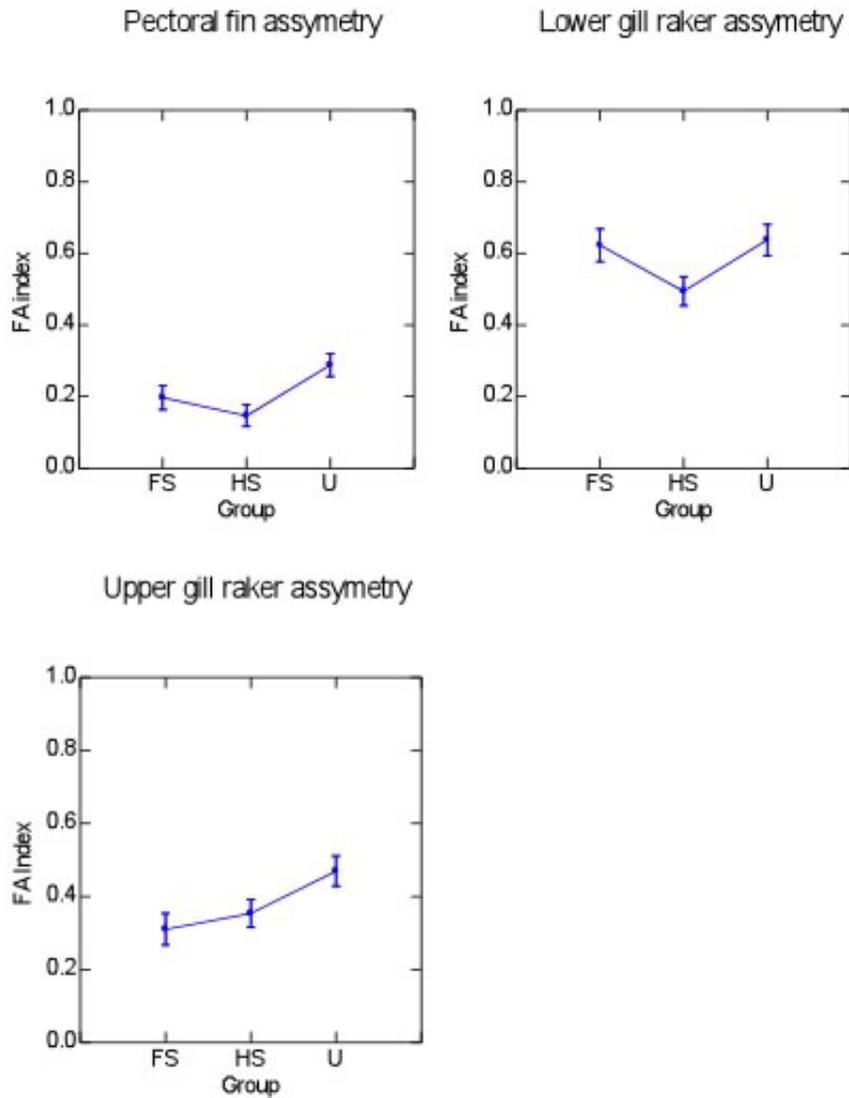


Figure 2. Mean size-corrected fluctuating asymmetry (FA) indices for three traits measured in 1998-brood juvenile chinook salmon at UWH in spring 1999 (mean FL 87.2 ± 0.3 mm). FS, full-sib mated; HS, half-sib mated; U, unrelated.